NATURAL HONEY AS A CRYOPROTECTANT TO IMPROVE VIABILITY OF VITRIFIED BOVINE OOCYTES

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ABSTRACT

Sucrose is commonly used in oocyte/embryo vitrification media. Natural honey is a mixture of 25 sugars (mainly fructose and glucose) having beneficial effects on cell viability. The main objective of this study was to investigate if natural honey can be used as a cryoprotecting agent (CP) in vitrification medium to improve the viability of vitrified-warmed bovine oocytes. The first study was conducted to investigate the dehydration capability of natural honey compared with sucrose, and to determine the proper concentration of honey-based medium and the optimum time for sufficiently safe dehydration of bovine oocytes. Matured cumulus-oocyte complexs (COCs) were denuded and introduced individually into different concentrations (0.25, 0.5, 1.0, 1.5 or 2.0 M) of honey and sucrose-based medium followed by rehydration in control media (TCM). Video images were recorded during dehydration and rehydration, and oocyte images were captured at 12 time intervals to calculate oocyte-volume changes during dehydration and rehydration. Results demonstrated that, in honey-based media, the maximum oocyte shrinkage was achieved after 60 sec exposure in 0.25M, 0.5M and 1.0M concentrations; while at higher concentrations 1.5M and 2.0M, the maximum dehydration occurred at 30 and 20 seconds respectively. In sucrose-based medium, the maximum oocyte shrinkage was achieved after 60 sec exposure in 0.25 or 0.5M concentrations. However, at higher concentrations (1M, 1.5M or 2M), the maximum dehydration occurred at 30, 20 and 10 sec. For rehydration, oocytes dehydrated in honey or sucrose-based medium were able to regain their original volume within 60-120 sec. However, oocytes dehydrated in higher concentrations (2M honey, and 1.5M and 2M sucrose) were rehydrated back to their original volume within 20 sec. This study concluded that natural honey and sucrose caused similar cell dehydration. Only oocytes dehydrated in 1M honey-based media reached maximal dehydration after 60 sec and equally regained original

volume. Therefore, 1M of honey-based medium is suggested for sufficient and safe oocyte dehydration during vitrification.

The second study was conducted to determine in vitro maturation (IVM), in vitro fertilization (IVF) and embryonic development of bovine oocytes vitrified in honey-based vitrifcation media. In Experiment 1, bovine COCs were randomly distributed in control group (non-vitrified; G1), 0.5M sucrose group (second control; G2), and 0.5M, 1M and 1.5M honey groups (G3, G4 and G5 respectively). The COCs were exposed to equilibration solution 1 (VS1) for 5 min and to vitrification solution 2 (VS2) for 1 min, mounted on Cryotops® and plunged into LN_2. COCs were warmed in TCM and honey/sucrose medium at 38.5^{o}C for 1 min, washed, matured in vitro (IVM), denuded, and immunostained to evaluate maturation. Maturation rate was significantly higher (80.7%) in control group (G1) than in vitrified groups (56, 52, 55 and 51% in G2, G3, G4 and G5, respectively) ($P<.0001$), whereas there was no significant difference among the vitrified groups ($P>0.05$). In Experiment 2, bovine COCs distributed in control (not vitrified, G1) and vitrified groups using 1M honey and 0.5M sucrose (G2 and G3 respectively), underwent for IVM, IVF and in vitro culture (IVC) for 9 days. Cleavage rate was significantly higher ($P<.0001$) in the control group (74%, G1, n=183) than rates of vitrified groups (51% in G2, n=137; and 42% in G3, n=131), whereas no differences among vitrified groups ($P=0.0723$). Rate of blastocyst formation was significantly higher (34%) in G1 than in the vitrified groups ($P<.0001$); however, blastocyst formation rates in the honey group were significantly higher ($P=0.0026$) than in the sucrose group (13% and 3% respectively). Addition of natural honey (1.0M; or 21.7%w/v) in vitrification medium can safely and sufficiently dehydrate bovine oocytes during vitrification procedure. The vitrification of bovine oocytes in 1M honey improved their post-warming maturation abtility and embryonic development.

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DEDICATION

This thesis is dedicated to the memory of my brother Muftah Alfoteisy, who was martyred on August 22 nd, 2011 striving to obtain justice, dignity and freedom for the people of Libya.

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LIST OF ABBREVIATIONS

AAs: Amino acids

AI: Artificial insemination

ART: Assisted reproductive technologies

BO: Brackett-Oliphant

BSA: Bovine serum albumin

BVDV: Bovine viral diarrhea virus

$C0_2$: Carbon dioxide

cAMP: Cyclic adenosine monophosphate

COCs: Cumulus-oocyte complexes

CS: Newborn calf serum

CTN: Cryoprotectant toxicity neutralization

Da: Dalton

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's phosphate buffered saline

EG: Ethylene glycol

FAO: Food and Agriculture Organization

FSH: Follicle stimulating hormone

GV: Germinal vesicle

GVBD: Germinal vesicle breakdown

h: Hour(s)

ICSI: Intracytoplasmic sperm injection

IIF: Intracellular ice formation

IVC: *In vitro* culture

IVF: *In vitro* fertilization

IVM: *In vitro* maturation

IVPE: *In vitro* produced embryos

kDa: Kilo dalton

LH: Luteinizing hormone

LN_2: Liquid nitrogen

M: Molar (M/L)

MI: Metaphase I

MII: Metaphase II

min: Minute

mL: Milliliter

M_w: Molecular weight

n: Number

NaCl: Sodium chloridenm: Nanometer

O_2: Oxygen

OPS: Open pulled straw

PROH: Propylene glycol, also called 1,2-propanediol

RNA: Ribonucleic acid

RT: Room tempreture

sec: Second (s)

μl: Microliter

μm: Micrometer

vs.: Versus

VS1: Vitrification solution 1

VS2: Vitrification solution 2

CHAPTER 1: INTRODUCTION

Cryopreservation is a fundamental tool of assisted reproduction and to establish a germplasm bank. Gametes (spermatozoa and oocytes) and embryos from genetically important individuals among several species and breeds can be preserved for future maintenance and enhancement in animal breeding programs, and for human medical purposes [1-3]. Frozen semen is readily accessible; however, preservation of female gametes is important to complete the animal genetic resources that might be utilized in future to re-establish back any loss of animal breeds. The most inexpensive and simple technique for cell cryopreservation is vitrification, during which cellular structure and extracellular solutions form glass-like state [4]. One potential direction for *ex situ* preservation of female genetics is cryobanking of ovarian (cortex) tissue or oocytes through vitrification technique [5]. Successful vitrification of mammalian oocytes can provide numerous practical, economical and ethical benefits. In general, mammalian oocytes are difficult to be cryopreserved successfully due to their large size, low surface area to volume ratio, high water content and low hydraulic conductivity [6]. Furthermore, vitrification of oocytes is still a challenge because of their structural and molecular sensitivity to chilling [7-10]. In the past, successful vitrification of oocytes has been reported in many species including mouse [11], cattle [12], buffalo [13], rabbit [14] and human [15]. Vitrification of bovine oocytes has been constantly improving, but obtaining a satisfied rate of blastocyst formation is still a challenge [7]. In spite of the encouraging results of vitrifying bovine oocytes [16-18], numerous studies have reported an extremely low rate of blastocyst formation [7,19-21] indicating the need for optimization of vitrification procedure. The optimal vitrification procedure requires dealing with several damaging factors including osmotic shock, cytotoxicity and the detrimental effect of ice crystallization. The osmotic shock occurs from variation in the osmotic gradient between the

intracellular and extracellular solutions. Cytotoxicity is a result from using high concentrations of permeant-cryoprotectant agents (CPs) that are usually used in the vitrification procedure. Intracellular ice crystallization is a detrimental effect that is a result from residual intracellular free water during the vitrification procedure [18,22,23].

Sugar incorporation in the vitrification medium is one of many attempts aimed at minimizing the effects of factors such as osmotic shock, cytotoxicity and the intracellular ice crystallization that are suspected to limit the success of cell viability after warming [24]. The main role of adding sugar in the vitrification medium is to cause an osmotic gradient across the cell membrane. The osmotic increase in extracellular solution usually results in the outward movement of intracellular water that minimizes the formation of intracellular ice crystals, thus reducing the lethal freezing injuries [25,26]. In addition, sugar enhances viscosity of the intracellular solutes, due to water efflux, minimizing intracellular-toxic effects of the permeating CPs [27,28]. Furthermore, it has been reported that including sugar in vitrification solution could significantly improve the survival rate of vitrified bovine blastocysts and immature human oocytes [29,30]. Sucrose [30], trehalose [31] and lactose [32] have been used as cryoprotective additives in vitrification solutions. Amnong these, sucrose is the most commonly used [33]. Fructose and glucose have been used in the cryopreservation of sperm without any cytotoxic effect [34,35]. These monosaccharides have been proven to be more effective than disaccharides to dilute concentrated cryoprotectants during the recovery of frozen-thawed mouse zygotes and human oocytes [36]. Raffinose (polysaccharide) has also proven to be effective in increasing the embryonic survival and development after cryopreservation [33,37]. Furthermore, combination of two sugars (sucrose and glucose) in the vitrification medium has more effectively improved the survival rate of the vitrified bovine blastocysts than the addition of sucrose alone [30]. It was

indicated that sugars have different characteristics and contributions in cell cryopreservation depending on their type and mass, a fact that should be taken into account before developing or modifying vitrification solution [33,38]. Increasing the extracellular non-permeating concentration in cryoprotective medium can significantly reduce the required concentration of permeable CPs for intracellular vitrification [28,39]. Therefore, ideal vitrification solution should have a maximum sugar concentration in order to enhance cell dehydration, thus minimizing the quantity of intracellular permeant CPs while not exceeding the hyperosmotic-tolerance limit of oocytes [28]. In this way, sugar alleviates high concentrations of penetrating CPs, and thus decreases their toxicity [40,41].

In this thesis, "natural honey" is being studied to determine its prospective use as a non-permeating CP in vitrification medium as an attempt to improve post-thaw viability of vitrified bovine oocytes. Honey is a natural combination of at least 25 sugars, mainly fructose and glucose. Sugars in honey account for approximately 95 to 97 percent as dry matter [42-44], and some of these sugars have been widely used in cell cryopreservation (as mentioned above). Honey also contains other bioactive constituents, beside dominant sugar components, mostly present in trace amounts [44,45]. The composition of honey (at least 181 components) including wide range of sugars, amino acids, organic acids, enzymes, vitamins and minerals, allows honey to provide numerous nutritional, biological and pharmacological effects in living cells[45-47]. For instance, some of these bioactive components in honey have been found to possess antioxidant, anti-toxicity, anti-inflammatory, anti-mutagenic, anti-cancer and anti-microbial properties in living cells [42-45,47-53]. To the best of our knowledge, natural honey has not been yet studied as an additive in cryopreservation media. In this study, it was presumed that the use of natural honey in vitrification medium as a non-permeant CP can render the cryoprotective

functions of sugars, improving post warming viability of oocytes and subsequent embryonic development. For instance, honey contains wide variety of amino acids [43-45,54]. Some of these amino acids have been successfully used as non-permeant CP to cryopreserve mammalian cells including sperm and oocytes [19,55,56]. For instance, it was demonstrated that adding glutamine (one of the amino acids in honey) into vitrification medium has improved maturation ability of vitrified immature bovine oocytes [19]. Furthermore, such a wide variety of components in honey have been proven to serve as antioxidants, including phenolics, peptides, organic acids (i.e. gluconic acid being predominant organic acid), vitamins (i.e. vitamin C being the most dominant) and the combined activities of the enzymes, like glucose oxidase, catalase and peroxidase. These antioxidatnt components in honey possess synergistic interactions (as group) resulting in a stronger antioxidant capacity than antioxidant effects resulted from an individual antioxidant component [47,57].

In first study, the dehydration ability of bovine oocytes (COCs) in different concentrations of honey and sucrose was compared. In second study, the effect of natural honey and sucrose, as non-permeant CPs in vitrification medium, on post-warming IVM, IVF and embryonic development of vitrified bovine oocytes was determined.

CHAPTER 2: LITERATURE REVIEW

2.1 *Conservation of animal genetic resources*

2.1.1 Introduction

For decades, animal producers and scientists have been facing the task of maintaining biological diversity and genetic stability in various species. Animal breeds possessing the endangered status and/or potential of genetic loss due to intensive genetic selection (e.g., high-producing cattle), deserve preservation. There are two basic methods to maintain the genetic resources of an animal species. One method is in situ conservation where the live animals are kept and maintained under natural environment. The second method is ex situ "cryopreservation" whereby the genetic material of an animal is preserved by subjecting sperm, embryos, oocytes or reproductive tissue to cryogenic temperature in order to suspend their biological activity. The metabolism in living cells is completely arrested at ultra low temperatures (-196 °C), that permits long-term cell cryopreservation. In order to bring back their function, the cryopreserved cells must then be warmed to the physiological temperature. Cryobiological studies have led to elucidate the events occuring during the cell freezing, and how the adverse effects can be overcome or minimized for successful cryopreservation [58-60].

2.1.2 Methods of gamete cryopreservation

The methods of cryopreservation of reproductive cells (i.e., sperm, embryos or oocytes) and tissues (i.e., testicular or ovarian) are constantly improving and numerous techniques have been developed to enhance the animal genetic resource banking human reproduction. However, except bull sperm, the post-warming viability of gametes using current methods of cryopreservation is still limited and need to be improved [61]. The cryopreservation techniques

regardless of biological variability between cell types or species are categorized into two methods, including slow freezing (with controlled programming) and vitrification. These two basic approaches of cell cryopreservation were designed to avoid the formation of lethal intracellular ice crystals during cooling or warming as well as the osmotic injury as water moves across the cell membrane and the toxicity of CPs [58].

2.1.2.1 Slow freezing method

Cryopreservation by slow freezing is a common method. It can be defined as a procedure where a sample is slowly frozen, by initiating extracellular ice nucleation by seeding, resulting in osmotically withdrawal of intracellular free water causing cell dehydration before freezing [62]. The slow freezing technique was first developed, in 1971, for cryopreservation of embryos in mouse [63-65] and few years later the technique was well established in sheep [66] and cattle [65] as well. In this technique, samples (oocytes or embryos) are gradually exposed to the culture medium with relatively low concentration of permeating CPs, which minimize mechanical damage to cells by preventing formation of intracellular ice crystals and they also strengthen cellular membranes. The commonly used CPs are glycerol or DMSO in a concentration ranges 1.0 to 1.5 M for oocytes or embryos. There are other common CPs used individually or in combinations. These CPs include permeating CPs such as EG and propylene glycol [67,68] and non-permeating ones such as sucrose [69], glucose, or fructose [70]. The cells/tissues are then loaded in small volumes into mini-plastic straws (often 0.25 ml), cooled to about -5 to -8 ˚C and are kept at this temperature for several minutes (e.g. 10 min) for equilibration. After, the extracellular solution in the straw of the sample is seeded manually (by touching the outer side of the straw with a piece of ice or very cooled metal like forceps) in order to initiate ice crystallization of the extracellular solution of the sample, causing cell

dehydration prior to freezing of intracellular matrix. After that, straw is cooled slowly to temperature point between -30 and -65 °C (depending on the protocol) at a rate of 0.3 to 0.5 °C/min. When germplasm (embryos or oocytes) are cooled to the desired temperature, the straws are immediately immersed into LN_2 and stored in the cryobank [71,72].

2.1.2.2 Vitrification method

Vitrification is a method where high concentration of CPs increases sample's viscosity to the point where the molecules become immobilized, and the sample is cooled using ultra-rapid velocity to ultra low temperature (-196°C) and cells/tissues are solidified to a glass-like state [73]. In 1985, Rall and Fahy were the first to use vitrification method simply by plunging the sample into liquid nitrogen (LN_2) at −196 °C [4]. The word vitrification came originally from the Latin word "Vitreous", which means glass. Glass transition, which prevents formation of intercellular ice crystals, happens as a consequence of thermodynamics. The permeating CPs used in this method enter the cell rapidly and form hydrogen bonds with intracellular water molecules, depressing the freezing point lower -120 °C, the glass phase temperature. Increasing the viscosity and cooling rate, or decreasing the volume will increase the probability of the sample vitrification [73]. Vitrification is a cost-effective option that can be done even under field conditions with no need of sophisticated equipment to manage the cooling rate as in slow freezing method. Also, vitrification is a good alternative for the use in various settings often encountered with wildlife species, such as zoos, field work in remote sites and poorly equipped locations [23].

2.1.3 Role of water in cell cryopreservation

Water accounts for up to 85 % of living cells (i.e. it depends on the cell type) and plays the most critical role in cell cryobiology. Cellular metabolism stops when all water in the system is frozen, since water is the major component of all living cells and must be available for the chemical processes of life to occur. A proper management of the intercellular water during cell freezing and thawing procedures is very important to avoid freezing damage that results in improvement of cell viability. For example, in the slow-cooling method, freezing occurs externally to the cell before intracellular ice begins to form. As ice is forming, water is removed from the extracellular environment and an osmotic imbalance occurs across the cell membrane leading to water migration out of the cell. The increase in solute concentration outside the cell, as well as intracellular, can be detrimental to cell survival. If the intracellular water remains inside the cell, damage to cell organelles will occur due to formation of ice crystals and recrystallization during warming [60,74].

2.1.4 Semen cryopreservation

Since the pioneer work on sperm cryopreservation by British scientists in 1950s [75,76], semen became the most practical tool of storing germplasm due its abundant availability and the simplicity of application [77,78]. Frozen sperm could be introduced back into existing populations any time up to decades or even centuries. The stored frozen-thawed semen from genetically superior males of threatened livestock breeds could be used for artificial insemination (AI) or in vitro fertilization (IVF) and has the potential to protect existing diversity and maintain heterozygosity [79,80]. In addition, it is possible to reconstruct animal breeds from semen through a series of back-cross generations. However, oocyte is still required to produce an

embryo in case of the loss of a breed. This suggests the need for successful cryopreservation of female germplasm (oocytes or ovarian-tissue fragments) along with semen and embryos to gain complete genetics of the original breed in order to reconstruct the target-breed population [81]. In the recent decade, semen of wide variety of mammalian and avian species have been cryopreserved successfully [78]. Furthermore, it was reported in 1998 that more than 260 million doses of bovine semen were produced (out of which 95% were deep-frozen). The frozen semen is used for insemination of one-fifth cattle population across the world [82]. However, the cryopreserved semen is commonly collected from genetically superior males and used in AI worldwide, which might narrow the available genetic diversity leading to loss of genetic variation over time [83-85]. In addition, the protocols currently used to cryopreserve bovine semen are still not optimal to be applied across other farm animal species [86]. Therefore, efforts have been made to optimize the semen cryopreservation protocols in other species such as goat [87], equine [88,89], and sheep [90].

2.1.5 Embryo cryopreservation

Cryobanking of genetic materials from bovine breeds by using cryopreservation of embryos is another important strategy since it enables conservation of complete animal genetics. It is also a valuable bio-security tool for reconstruction of animal breeds in case of contingency epidemic disease and other catastrophes [91]. The first offspring by transferring cryopreserved embryos was produced in 1972 [63], and the successful live birth resulting from in vitro fertilization (IVF) of cryopreserved oocytes was reported in 1976 [92] followed by successful vitrification in 1985 [4]. Since then, considerable successful numbers of experiments and scientific investigations have been performed in order to optimize the methods of embryo

cryopreservation and transfer. As a result, hundreds of thousands of embryos (whether in vivo derived or in vitro produced) have been successfully cryopreserved and transferred [93]. For instance, in 2002, more than 220 000 cryopreserved bovine embryos were transferred [94], and in human, in 2004, more than 12 000 babies were born as the result of cryopreserved embryo transfer [95,96]. These sustained efforts in embryo cryopreservation provide practical and economic benefits that will positively impact animal breeding programs and assist conception in humans [97].

2.1.6 Ovarian tissue cryopreservation

Another possible strategy for maintaining animal genetic diversity is to store female gametes by cryopreservation of the entire ovary, isolated follicles or ovarian cortex, i.e. the area where the resting pool is located. The mammalian ovarian cortex contains mainly primordial follicles followed by primary follicles and a minor number of antral and secondary follicles which remain viable for several hours after the death of an animal [98]. Such follicles could be recovered after the animal's death and successfully cryopreserved in order to utilize in future by xenotransplantation [99] or in vitro culture [100] to obtain mature fertilizable oocytes. Cryopreservation of ovarian tissue would only preserve the oocytes in primordial and primary follicles. The antral follicles containing immature oocytes at germinal vesicle (GV) stage, do not survive cryopreservation [101]. The oocytes in the primordial and primary follicles are much less susceptible to cryopreservation damage because they are small (i.e. in cattle, oocyte size in primordial and primary follicles averages: 29.7 and 31.1 µm respectively [102]; and in human, oocyte size in primordial and primary follicles averages: 36 and 42.1 µm respectively). Also, oocytes enclosed in such small size of follicles are not well-developed, with few organelles, no

zona pellucida, and are almost metabolically quiescent and undifferentiated [103,104]. However, preservation of ovarian tissue as a strategy to cryopreserve germplasm is still a challenging task because of cell variation that ovarian tissue contains (oocytes, granulosa cells, endothelial cells and specific extracellular matrix components) and prolonged phase of follicle/oocyte growth and development [104,105]. The challenge of a successful *in vitro* culture procedure to produce mature oocytes from primordial follicles includes *in vitro* culture of small fragments or slices of the ovarian cortex in order to activate the primordial follicles to be developed to primary, secondary, preantral and ultimately antral follicles capable to produce oocytes[102]. Regardless of the fact of cryodamage and the complexity of in vitro tissue culture procedure, numerous studies in many species have assured the potential success of this technique [106,107]. Oocytes in primordial follicles are very small (i.e. primordial oocyte in newborn mice is approximately 20 μm in diameter [108]) and able to tolerate cryopreservation very well, but there is still a challenge in producing in vitro mature oocytes from these primordial follicles. The process of folliculogenesis takes approximately 4 to 5 months, during which period the follicles/oocytes and surrounding somatic cells go through a series of changes that eventually result in the development of a large antral follicle, capable of reaching MII stage [109]. Recently, a two-step *in vitro* culture system has been developed for bovine ovarian tissue to grow primordial follicles to reach secondary stage, and these secondary follicles were then isolated for further growth in vitro to obtain antral follicles with oocytes larger than 100 μm [110]. This bovine follicular development rate in vitro is accelerated compared to the in vivo rate, and found to be consistent with other studies in sheep [111] and humans [112]. A shorter growth time was observed in vivo compared with in vitro for preantral follicles to reach the antral stage. This raises the question

whether or not this is a normal follicular development capable of producing a healthy mature oocyte.

The carrier devices that commonly used for vitrifying ovarian-tissue fragments are plastic straws, cryotop, cryoloop, electron microscope grid and cryovials. These carrier devices have limited cooling rates comparing with carrier devices used for vitrifying oocytes because they have thick walls, which probably negatively influence the cooling rate. Recently, several studies have developed carrier-less volume systems including solid-surface vitrification and minimum drop size [23]. These carrier systems could reduce the vitrification solution volume, and thus increasing the cooling and warming rates for vitrifying ovarian-tissue fragments [23].

2.1.7 Mammalian oocyte cryopreservation

Oocytes (esp. immature COCs) are different from sperm and embryos in tolerance of cryopreservation procedure due to morphological differences. The mammalian oocyte is large, i.e. a mature oocyte ranges from 75 to140 μm in diameter depending on the species [113], which is three to four times larger than a sperm cell, thus substantially decreasing the surface-to-volume ratio and making them very susceptible to chilling and intracellular ice formation [114-117]. Oocyte cryopreservation is an attractive strategy since it does not have ethical considerations and legal objections as embryo freezing [118]. Oocytes obtained by in vivo pickup or at abattoir can be cryopreserved for long periods of time for subsequent IVF and embryos production. Banking of mammalian oocytes would expand the diversity of gene pool, provide more options for assisted reproductive technology, preserve female genetics after unforeseen death, and avoid controversy surrounding the preservation of embryos [5,119]. Oocyte cryopreservation would be beneficial for global exchange of germplasm as semen, since

it avoids injury and potential risks of disease transmission involved in transportation of live animals [7]. The first attempt in cryopreservation of mammalian oocytes was in 1958 [120]. In 1977, the first live births of mice pups from mature oocytes cryopreserved by conventional freezing and stored in LN_2 reported [121].

2.2 *Oocyte vitrification*

2.2.1 Characteristics of mammalian oocytes

Once the female reaches puberty, a cohort of primary follicles (oocyte) is selected in each estrus cycle which continues to develop to the point where they become dominant. As a result, one (cow and mare) or several follicles (sow and bitch) are ruptured and oocytes are ovulated, whereas the rest of the follicles regressed and degenerated before oocyte ovulation. Prior to fertilization, an oocyte must reach the metaphase II (MII) stage of maturation, otherwise, probability of fertilization is very low [122]. Also, during folliculogenesis as the oocytes grow, oocytes need to synthesize and accumulate large quantities of mRNA and proteins in order to continue for meiosis, fertilization, and embryonic development procedures [123]. Therefore, any deficiencies in the entire supporting system during IVC diminish synthesis of some of these essential components resulting in suboptimal oocyte quality [123].

2.2.2 Principles and rationale of oocyte vitrification

The vitrification method was initially designed to eliminate the formation of intra- and extracellular ice crystals. The whole idea is to minimize or eliminate ice formation and transform cells into an amorphous glassy state, which is less damaging than ice crystals. Vitrification of oocytes, like other cells, requires brief exposure to high concentrations of CPs during the

procedure. The CPs could provide dehydration either by entering the cells and binding with water molecules (permeating CPs) or by remaining largely out of the cell and drawing out the intracellular water by osmosis (non-permeating CPs). In rapid cooling method or vitrification the rationale of using higher concentrations of permeating CPs is mainly to decrease the freezing point of the intercellular solutes in order to prevent potential damage from high electrolyte concentrations and intracellular ice crystallization of vitrified oocytes [73,124]. However, a very high concentration of CPs damages the cells by causing them to be extremely susceptible to cytotoxicity and osmotic shock [7]. The osmotic shock is more critical to cell survival than inherent toxic consequences of exposure to concentrated solutions [125].

2.3 Cryoprotectants (CPs)

Cryoprotectants (CPs) are classified into permeant and non-permeant CPs depending on their cell penetration and molecular weights (Table 2.1).

Table 2.1 Classification of cryoprotectant used in cell/tissue cryopreservation (adapted from [126]).

Permeant CPs, low molecular weight (M_w)	**Non-permeant CPs, high molecular weight (M_w)**	
Cryoprotectants with M_w < 100 Da.	**M_W ≥180 to ≤ 594 Da**	**M_W > 1000 Da (KDa)**
Ethylene glycol, EG (62.07)	**Glucose, dextrose (180)**	**Hydroxyethyl starch, HES (1.35)**
Propylene glycol/ 1,2-Propanediol (76.09)	**Fructose (180.16)**	**Polyethylene glycol, PEG (1.45)**
1,3-Propanediol (76.09)	**Lactose (342.30)**	**Polyvinylpyrrolidone, PVP(10-40)**
Dimethyl sulfoxide, DMSO (78.13)	**Sucrose (342.30)**	**Polyvinyl alcohol, PVA (30-70)**
Glycerol, or 1,2,3-Propanetriol (92.09)	**Trehalose (378.33)**	**Ficoll®70 (60- 80)**
Butylene glycol (2,3-butanediol) (92.12)	**Raffinose (594.51)**	**Dextran (>500)**
Acetamide (59.07), (most toxic CP)		
Formamid (45.04), (most toxic CP)		

2.3.1 Permeating CPs

The permeating CPs mainly penetrate into the cells and should have low toxicity in order to be biologically acceptable [127]. When cells are exposed to a permeating CP, they will momentarily shrink and then swell due to water movement across the membrane. Shrinkage is caused by the initial hyper osmoticity of the extracellular solution and the fact that cells are much more permeable to water than to cryoprotectant. Shrinkage of cells stops when the efflux of intracellular water and the influx of extracellular cryoprotectant reach an equilibrium state. Cryoprotectants will enter the cells at a rate dependent on their membrane permeability, the temperature, the surface area to volume ratio of the cells, and the gradient between intracellular and the extracellular CP concentration [128].

The permeating CPs basically forms hydrogen bonds with intracellular water molecules and lower the freezing point of solution [41,126,129]. The permeating CPs also increase the total concentration of intracellular and extracellular solutes in the system eliminating the amount of ice formation at any given temperature. The permeant CPs include glycerol (the first CP used successfully in cryopreservation of fowl spermatozoa [75]), dimethyl sulfoxide (DMSO; which has the advantage of more rapid penetration into most cells [130]), ethylene glycol (EG; which have greater permeability [131]), polyethylene glycol (PEG) and 1,2-propanediol (also called propylene glycol, PROH) have lower molecular weights [41,126].

2.3.2 Role of permeating CPs in cell cryopreservation

The protective properties of cryoprotectants are related to their ability to lower the freezing point of the solution in a concentration-dependent way and also to their chemical

properties. In cell cryopreservation, permeating CPs enter the cells rapidly and form hydrogen bonds with intracellular water molecules preventing (or minimizing) water-to-water hydrogen bonding that is the basis of forming the lethal ice crystals within the cells. In addition, CPs prevent cryopreserved cell from potential damages caused by high salt concentration due to dehydration of the cells during slow freezing [126]. The search for suitable cryoprotectants and their appropriate concentrations has been a priority in all the freezing protocols to have maximum cryoprotective effect with minimum osmotic and toxic effects in post-warming cell viability. There is no ideal cryoprotectant agent used so far in cell cryopreservation to be free of toxicity [132].

2.3.3 Nonpermeating CPs

Nonpermeating CPs cannot enter the cell due to their large size molecules. They include macromolecules or polymers often added in cryopreservation medium to reduce the amount of intracellular cryoprotectants required for vitrification to minimize the toxicity of the solution and to withdraw water from cells. Polymers protect zona pellucida against cracking. The majority of solutions used for oocyte vitrification contain a macromolecular component of bovine serum albumin (BSA) or fetal calf serum (CS). Other macromolecules used in vitrification media include PEG, PVP, Ficoll, and PVA. Other non-permeant CPs, used in vitrification medium to improve the viability of cryopreserved tissue, include sucrose, trehalose, raffinose, lactose, glucose and fructose, as well as some proteins and lipoproteins [1]. In addition, it was shown that a mixture of permeant CPs and saccharides can reduce the toxic effect of CPs [27,30,133].

2.3.4 The use of sugar in cryopreservation

Sugar molecules such as sucrose, terhalose, glucose, fructose, ficoll (hydrophilic polysaccharides), and raffinose have been used in cryopreservation in order to counter the osmotic shock and reduce the cytotoxicity which decrease viability of frozen-thawed cells [33]. The inclusion of sugars as non-permeating compounds in the diluting solution is to reduce the osmotic gradient between the intracellular and extracellular solutions. The first study conducted on the addition of sucrose as a non-permeant CP aimed to reduce of osmotic shock during thawing of cryopreserved embryos [134]. The rationale, described earlier [24], was to make equal osmolality between the extracellular diluting solution and the intracellular solution by the addition of sugar (usually sucrose). Therefore, the cell experiences no net influx of water when the concentrated cryoprotective additive solution is diluted and the osmolalities of both intracellular and extracellular solutions become equal.

The occurrence of intracellular ice formation during freezing can be minimized by dehydration of cells [74]. The damage due to intracellular ice formation cannot be totally prevented, but it can be significantly minimized if the necessary degree of cellular dehydration and CPs permeation are met while avoiding toxic and osmotic injury [135]. Cryopreservation methods (including slow equilibrium cooling [25], rapid non-equilibrium cooling [26,136] or vitrification) have been developed by adding sucrose to the cryoprotective solution to make cells shrink (in response to higher osmotic pressure of the extracellular solution) to enhance cell dehydration before freezing [55].

Disaccharides such as sucrose, trehalose [31] and lactose [32] have also been used as cryoprotective additive solutions for the same purpose. In vitrification solutions, disaccharides

are commonly used in vitrification of oocytes and embryos in various species [33], e.g. mouse [137], sheep [138], cattle [139], horse [140], rabbit [14], buffalo [141] and human [142] embryos; and mouse [11], cattle [12], buffalo [13] and human [15,143] oocytes. Raffinose (polysaccharide) with an equal concentration has proven to be effective in increasing the survival rate after exposure to either a monosaccharide or a disaccharide, suggesting that the damage to the cell may be further reduced, depending on the combination of saccharides added to the vitrification medium [33]. It has been concluded in another study that the vitrification medium with the addition of both sucrose and glucose effectively improved the survival rate of the vitrified bovine blastocysts than the addition of sucrose alone [30]. A recent study investigated the effects of different sugars (i.e. glucose, sucrose, or a polysaccharide) as nonpermeant CP in vitrification media on *in vitro* maturation of vitrified-warmed immature (GV) porcine oocytes. As a result, the porcine oocytes that were vitrified in vitrification medium containing sucrose as nonpermeant CP had higher maturation rate compared to oocytes vitrified in glucose-based medium [144].

However, glucose is recognized as an essential component in the majority of culture media in oocyte maturation and embryo culture media [145]. Monosaccharides such as fructose and glucose have been used in cryopreservation of sperm, and they have no cytotoxic effects [34,35]. Moreover, monosaccharides have more effective osmotic buffers than disaccharides to dilute the effect of concentrated cryoprotectant during the recovery of frozen-thawed mouse zygotes and human oocytes [36]. Monosaccharides, especially fructose, had a better effect on semen quality than disaccharides and trisaccharides in red deer and dog sperm cryopreservation [146,147]. Another study [147] concluded that disaccharides (including sucrose and trehalose) decreased dead sperm and the damaged acrosome rate in dog sperm without promoting post-

thaw motility. However, monosaccharides (fructose) improved both the intact acrosome rate and motility. Other researchers showed that the effects of different sugars on mouse sperm cryopreservation depended on their mass concentration, but not in their molar concentration [38]. Monosaccharides can be mixed more easily and efficiently even with concentrated solutions of CPs because they have a lower viscosity than disaccharides [36]. It was discussed in a review [148], using monosaccharides may assist in the development of methods of improving oocyte cryopreservation. In vitrification methods, the formation of ice crystals is almost eliminated both within the cells being vitrified (intracellular) and in the surrounding solution (extracellular). The presence of sugars (like trehalose, glucose, or raffinose) intracellular and extracellular during vitrification of mouse oocytes has been found to alleviate the high concentrations of penetrating CPs, and thus minimizes their toxic effects [37].

2.3.5 Toxic consequences of CPs

Cytotoxicity of CPs is a fundamental limiting factor for the successful cryopreservation of living systems in both slow freezing and vitrification [149]. The mechanisms of cytotoxicity due to high concentrations of CPs in vitrifying solutions have not been elucidated [150]. The cytotoxicity of CPs has been shown to increase with time, temperature and concentration. For instance, the toxic effect of CP solution in skin tissues was significantly reduced at low temperature, i.e. of 4 °C [151]. Room temperature is a preferable temperature for cell cryopreservation procedure, and low temperatures could unnecessarily extend the duration of time required for cryoprotectants to permeate the cells. At higher temperatures, certain cryoprotectants are suspected to have toxic effect [152]. Several studies [153-157] have demonstrated variation in cryoprotectants' cytotoxic effects in living cells. For instance, EG and

glycerol were less toxic than PROH, DMSO, and formamide [126,153]. In the methods of cryopreservation, such as ultrarapid cooling [158] or vitrification [4,73,124], the higher concentrations of permeating CPs (6 M) are often used in order to decrease the freezing point of the solution and prevent oocyte damage from high electrolyte concentrations [73]. However, a very high concentrations of CPs result in damaging the cells by causing them to be extremely susceptible to cytotoxicity and osmotic shock [7]. Cytotoxicity is especially critical in vitrification, which requires much higher concentrations of CPs, and there is variability between cell types and species. The toxic effects of cryoprotectants destruct the cell viability much more than their osmotic stress because their toxicity and protective efficiency are relatively based on their intrinsic nature of chemical reactions than osmotic pressure [159]. The cytotoxicity of CPs is a continuous concern in cell vitrification procedure, which requires the use of much higher CP concentrations. Therefore, successful vitrification requires a full consideration about the chemical nature of CPs used in the procedure, the exposure time, the temperature, and concentration of CPs because these factors play a critical role in the post-warming cell viability [160].

2.3.6 Combination of CPs and reduction in cytotoxicity

Different strategies have been used to decrease the toxic harmful effects of high concentrations of CPs without weakening their cryoprotection ability [149]. One is a combination of relatively low concentrations of CPs in order to obtain a workable concentrations of total vitrification solutes [161] to diminish the CP-associated toxicity [162]. A mixture of cryoprotectants could reduce the toxic effects of high concentrations of other CPs in the vitrification solution [163]. It was found that mixing two or more of CPs in vitrification solution

could cause synergistic interactions between CPs. These interactions of a mixture of two CPs lead to less cytotoxic than the addition of the individual CPs [163]. Another strategy for reducing cryoprotectant toxicity is termed as cryoprotectant toxicity neutralization (CTN). It was first convincingly introduced in 1971 [133] demonstrating that addition of the amides with a given concentration of dimethyl sulfoxide (DMSO) can relatively block its cytotoxicity effect. However, recent studies investigated a mixture of commonly used CPs with amides reported a lack of evidence to support that CTN can be obtained by inclusion of the amides agents [150]. Furthermore, the combination of dextrose (glucose) with DMSO was reported to prevent irreversible binding of DMSO to proteins, and thus reducing adverse cytotoxicity effect [164].

The efficiency of the CPs depends mainly on their solubility in water at all temperatures relevant to freezing and on the lack of toxicity. The latter property is important because it generally determines which chemical agents are permeable to the cell membrane to serve as useful CPs and which are not. Therefore, cryoprotectant toxicity can be considered as the most critical limiting factor to achieve a successful cryopreservation by either slow freezing or vitrification and for the scope of cryobiological protocols [129].

2.4 Intracellular ice crystallization

The understanding of intracellular ice formation (IIF) is important for cryopreservation and cryosurgery techniques. In the cryopreservation technique, vitrification is designed to accelerate cooling rate to exceed rate of IIF in order to minimize the ice crystals, thus avoiding cryo-damage [4,129]. The size of intracellular ice crystals generally ranges from <0.1 nm to several nanometers [165,166] and the freezing point depression of intracellular ice was found to be approximately 1 to 10°C. Formation of intracellular ice crystals is characterized into three

different levels including the formation of primary crystals, secondary crystals or higher level [28]. Once ice crystals are formed, the rate of growth depends on the function of temperature. The lower the temperature is, the slower the ice crystallization rate occurs. Rapid cooling rate will result in many small ice crystals because no sufficient time for those nucleated crystals to grow largely, which leaves liquid water still available to be nucleated at a lower temperature. Therefore, the slower cooling rate leads to formation of the larger intercellular ice crystals, a fact which mainly harms the cell organelles including cytoplasmic lipid droplets and meiotic spindle of oocytes [167].

2.5 *Vitrification and subsequent osmotic shock*

In vitrification method, cells require brief exposure to high concentration of CPs that might cause extreme volumetric changes known as "osmotic shock". One function of the CPs in vitrification procedure is to provide cell dehydration either by entering the cell and binding with intracellular water molecules causing dehydration (permeating CPs) or by remaining largely out of the cell and drawing out the intracellular water by osmosis (non-permeating CPs). Improper addition or removal of the CPs in vitrification or warming procedures often results in osmotic shock to the cells; a fact which is critical as the inherent toxic effect of CP regarding cell survival and viability [125]. It was found that osmotic stress is detrimental to bovine oocytes, particularly GV stage compared to MII, and must be considered when developing or optimizing cryopreservation procedures [168]. The osmotic shock injury to frozen-thawed cells can be controlled by the inclusion of non-permeating (usually sugars) compounds in the diluting solution to reduce the osmotic gradient between the intracellular and extracellular solutions [134].

2.6 *Oocyte stage and tolerance of cryoinjuries*

Mammalian oocytes are cryopreserved at different stages including at the germinal vesicle (GV) stage, and metaphase II (MII) stage [169]. However, the stage of nuclear maturation at which oocytes are cryopreserved impacts their post-warming viability and developmental competence [170]. Therefore, a decision on whether to cryopreserve oocytes at the GV or MII stage depends on the consideration of some characteristics related to chilling sensitivity of each stage.

2.6.1 Germinal vesicle (GV) stage

Oocytes in small (<5 mm) follicle in ovaries obtained from abattoir are at the stage of germinal vesicle (GV), in which the condensed chromosomes are within the nucleus. At this stage, oocytes potentially tolerate the chilling impact because of the absence of the meiotic spindle and condensed chromosomes. However, the oocytes at GV stage have less membrane permeability with tight gap junctions and compact cumulus cells which might retard exchange movement between the permeant CPs and intracellular water, leading to inadequate dehydration and thus prone to cryoinjury. Even though such factors are considerable, it was demonstrated that bovine COCs at GV stage were able to survive vitrification with higher rates of subsequent embryonic development than MII oocytes [170]. This indicates that GV stage might be more feasible to be vitrified since they do not have the meiotic spindle or other structures sensitive to low temperature.

2.6.2 Metaphase II (MII) stage

Oocytes at the metaphase II (MII) stage of maturation can also be cryopreserved. At this stage, the oocyte has expanded surrounding cumulus cells and microtubules form the spindle apparatus [7]. Abnormalities in the meiotic spindle can cause failure of normal fertilization and embryo development because spindle is a fundamental tool for completion of meiosis, formation of second polar body, movement of the pronuclei, and arrangement of the first mitotic spindle [171-173]. Therefore, cryodamage of matured oocytes potentially result in chromosomal dispersion, incomplete fertilization, and failure in developmental competence [7,171].

2.6.3 Quality of oocytes and vitrification

Oocyte quality is a crucial base to produce embryo quality [104]. The evaluation of oocyte quality before cryopreservation is a fundamental aspect to achieve good outcomes. The criteria for selecting a good quality oocyte whether derived by in vivo pickup or from ovaries collected from slaughterhouse should include uniform cytoplasm, round in shape, the size, healthy appearance with expanded cumulus cells and the absence of cytoplasm vacuoles or any abnormality (e.g. granular). Oocytes matured in vivo were found to exhibit a significant maturation rate and blastocyst formation compared to those matured in vitro [174]. Bovine oocytes that derived from large growing follicles (size ≥6 mm) exhibited significant improvement in *in vitro* maturation, fertilization and subsequent embryo development [7,175].

2.7 *The importance of cumulus cells in post-warming viability of vitrified oocytes*

One of various attempts to improve the post-warming viability of vitrified oocytes was to completely or partially remove the cumulus cells. This is assumed that cumulus cells retard the penetration of cryoprotectants, resulting in an unequal intracellular distribution of the cryoprotectant and inadequate cell protection.

However, the cumulus cells surrounding the oocyte are specialized to regulate the oocyte development**.** It has been found that a communication between oocyte and surrounding cumulus cells is critical for oocytes to obtain successful nuclear and cytoplasmic maturation [176]. Cumulus cells provide a complex microenvironment and nutritive products which are beneficial to sperm for their penetrating and fertilizing abilities. In addition, cumulus cells communicate with oocyte through gap junctions to provide essential factors for fertilization [176]. They play an important role in attracting, trapping and selecting proven sperm, and facilitating sperm capacitation, acrosome reaction and penetration. It was found that denuded oocytes face depletion of enzymes, maturation promoting factor (MPF) and mitogen activated protein Kinase (MAPK) that are required to support embryo development [176-178]. The gap junctions connect between the oocyte and granulosa cells and connect as well as between cumulus cells to grant network communication that are important for further development and fertility of the oocyte [179,180]. Also, pores in conventional gap junctions permit molecular exchange of substances possessing molecular weight less than 1 kDa, including sugars, amino acids, lipid precursors and nucleotides, by diffusion process. This is the most important route of entry to the oocyte and may be the only pathway for these nutritive substances [181].

In vitrification of goat oocytes, the addition of cysteine and cysteamine improved the *in vitro* maturation ability of denuded oocytes [182]. However, the detrimental effect of cumulus removal prior to IVF was observed in cattle. In a recent study in vitrification of immature (GV) or mature (MII), it was confirmed that cumulus cells are essential for post-warming viability of vitrified oocytes particularly when COCs are vitrified at GV stage. COCs (GV) have exhibited survival and embryonic developmental competent rates significantly higher than MII COC stage [170].

2.8 *Vitrification carrier devices and cooling rates*

There have been many carrier devices developed over time to increase the cooling and warming rates to avoid lethal effects of intracellular ice crystallization. These methods include the conventional carrier method of 0.25-mL straw vitrification which initially provided a fast cooling rate of 2,500 °C/minute and a warming rate of 1,300°C/minute [4]. Since then, many vitrification devices have been developed to increase rates of cooling and warming of the cells. These methods can be divided into two groups: (a) closed system like cryoTip [183], sealed pulled straw[184] and hemi-straw system [185], and (b) open system such as electron microscopic copper grids [186-188], thin glass surfaces [189], microdrop [18], open pulled straws [16], cryoloops [190], and cryotop [15,191]. The open system of carrier devices has an advantage that can increase the rates of cooling and warming of vitrified cells. Sample volume (i.e. an oocyte or embryo enclosed in vitrification solution) can be reduced to the minimum ($\leq$1μl) before vitrification, thus minimal sample volume results in a higher cooling velocity in cell vitrification [167,192]. Smaller volume allows better heat transfer, thus enhancing ultra-rapid cooling rates. For instance, the cooling and warming rates with the use of cryotop method

(with minimum volume) were significantly increased to reach ≥23,000 °C/min and ≥42,000 °C/min, respectively[183]. The ultra-rapid cooling and warming rates of these methods enabled oocytes to rapidly pass through a critical temperature zone. Since oocytes tend to be susceptible to chilling injury between 15°C and −5°C, extremely rapid rate of thermal changes reduces the chance of cryodamage to oocytes during cooling and warming cycles, and thus resulting in a higher post-warming viability [187]. The cryotop method [191] with implementing a minimal-sample size before vitrification offers at present one of the most efficient and preferred methods for oocyte vitrification [193].

2.9 Possible contamination by direct contact of LN_2

By using the "open vitrification system", oocytes are loaded on the strip with minimal volume of medium and the device is immersed immediately and directly into liquid nitrogen (LN_2). However, despite a high vitrification capability and potential usefulness in many practical applications, an open vitrification system brings the risk of infection hazards, such as bacterial or viral contamination to cryopreserved cells [94,194,195]. Many studies on in vitro produced embryos (IVPE) indicated a high possibility of pathogenic transmission [94]. For example, the introduction of BHV-1 and BVDV and some other viruses during IVPE and the association of these viruses with developed embryos have been demonstrated. Such viruses were found in follicular fluid and in COCs [196], and these COCs are the main source of vitrified oocytes that are used for producing in vitro production of embryos. The majority of tested micro-organisms including bacterial and viral agents that either in the form of association with germplasm or in pure cultures can survive storage in LN_2 (−196°C) [94], and even some of micro-organisms can tolerate concentrations up to 45% of some CPs without visible toxic effects [94].

In regard of avoidance of possible microbial cross-contamination that resulted from the direct contact of the LN_2, several methods of closed-system vitrification have been developed such as cryotip, cryohook or others. The idea in such devices was either by sealing the carrier or inserting it into large sterile plastic-sealed straws before their immersion in LN_2. In fact, the closed system method (cryotip) has failed to demonstrate the same efficiency in terms of contamination with the open system (direct contact) method such as cryotop [197]. Unfortunately, so far, the open system that has direct contact to LN_2 with potential risk of infection hazards is the indispensable option to achieve the required cooling and warming rates for very sensitive samples like oocytes [94]. Alternative devices have been developed to improve cooling/warming rates of closed system method (cryotip) is using a super-cooled liquid nitrogen (-210°C) [192,198].

2.10 Hypotheses

The overall hypothesis in this thesis is that "natural honey" as a bioactive mixture of mainly sugars can be used as a non-permeant cryoprotectant consolidating the common sugars used in vitrification medium to improve post-warming viability and subsequent embryonic development of vitrified bovine oocytes. The specific hypotheses are:

1. Natural honey-based medium has similar dehydration capability to bovine oocytes as sucrose.
2. The volumetric response of bovine oocytes during dehydration and rehydration in honey and sucrose based media is concentration-dependent.
3. Incorporation of optimal concentrations natural honey in vitrification medium can induce efficient and safe dehydration to bovine oocytes before vitrification procedure.
4. Vitrifying immature bovine oocytes in a medium containing an optimal concentration of natural honey improves their post-warming maturation, fertilization and embryonic development.

2.11 Objectives

The overall objective this study was to investigate the dehydration ability of natural honey as a non-permeant CP for vitrification of bovine oocytes. The specific objectives of this study were:

1. To investigate volumetric changes of bovine oocytes as response to the exposure time of different concentrations of honey and sucrose based media.
2. To compare the effects of different concentrations of natural honey with the control concentration of sucrose (0.5M) on the post-warming in vitro maturation of vitrified bovine oocytes
3. To study the effects of an optimal concentration of natural honey with comparison of the 0.5 M sucrose on cleavage and blastocyst rates of bovine oocytes following vitrification.

CHAPTER 3: NATURAL HONEY AS A NON-PERMEANT CRYOPROTECTANT: BOVINE OOCYTE MODEL

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3.1 Abstract

Sucrose is commonly used in vitrification medium to dehydrate cells in order to reduce the occurrence of fatal intracellular ice crystallization during cryopreservation. Natural honey is a mixture of 25 sugars (mainly fructose and glucose) having beneficial biological and pharmacological effects on cellular viability. This study was designed to determine if honey, in place of sucrose, can be used as nonpermeant cryoprotectant for vitrification of bovine oocytes. Denuded-matured oocytes were exposed to different concentrations of sucrose or honey (0.25, 0.5, 1.0, 1.5 or 2.0M), under microscope. Video images were recorded to evaluate oocyte volume change in different concentrations of honey and sucrose, and in rehydration media. Natural honey caused similar ($P>0.05$) cell dehydration as sucrose. In honey-based media, the maximum oocyte dehydration was achieved at 60 seconds after exposure in 0.25, 0.5 or 1M concentrations; and at 30 and 20 sec in 1.5 and 2 M concentrations, respectively. In sucrose-based media, maximum oocyte dehydration was achieved at 60 seconds after exposure in 0.25 and 0.5M concentration; and at 30, 20 and 10 sec in 1, 1.5 or 2 M concentrations, respectively. During rehydration, oocytes in all concentrations of honey or sucrose-based medium were able to regain their original volume within 60-120 seconds. However, oocytes dehydrated in higher concentrations of and sucrose (1.5 and 2M) rehydrated within 20 and 10 sec respectively. A significant relationship was found between time and ooplasm volume change ($P<0.05$), during dehydration and rehydration phases, in both honey and sucrose. The concentration of 1M honey

was equivalent to 0.5M sucrose in order to achieve sufficient and safe oocyte dehydration prior to vitrification.

Key words: Bovine oocyte, natural honey, sucrose, dehydration and volume.

3.2 Introduction

Vitrification is a promising cryopreservation technique during which intracellular and extracellular solutions become solid in a glass-like state [4]. This technique has been tried to cryopreserve oocytes and embryos in various species for the past decade [27]. However, the optimal vitrification procedures have not been met in achieving high cell viability rate after vitrification due to several major problems [2,7], including the possible formation of intracellular ice crystals, osmotic shock and cytotoxicity due to the high concentrations of cryoprotectants (CPs) [2,27,157]. The addition of sugars in cryopreservation medium is one of approaches to overcome the problems limiting the success of cell viability after warming [24]. Sugar being non-permeating CP causes an osmotic gradient across the cell membrane that enhances cell dehydration before freezing, in order to reduce the quantity of intracellular water. This reduction of the intracellular water decreases the chances of intracellular ice formation and minimizes lethal freezing injuries [25,26]. Also, sugar enhances viscosity of the intracellular solutes (i.e. decreasing quantity of penetrated CPs bound with intracellular water) minimizing the intracellular toxic effects due to the permeating cryoprotectants [27,28]. Furthermore, vitrification solution that includes sugars could significantly improve the survival rate of vitrified bovine blastocysts and human-immature oocytes [29,30].

Monosaccharides such as fructose and glucose have been used in cryopreservation of sperm without any cytotoxic effects [34,35]. Moreover, monosaccharides were proven to be more effective than disaccharides to dilute concentrated solutions during the recovery of frozen-warmed mouse zygotes and human oocytes [36]. Fructose has a better effect on semen quality than disaccharides and polysaccharides for the cryopreservation of red deer sperm [146]. Interestingly, disaccharides like sucrose and trehalose (not lactose) increase sperm viability and reduce damaged acrosome in dog sperm without promoting post-thaw motility [147], while fructose improved both the integrity acrosome and motility rates. Other researchers showed that the effect of different sugars during mouse sperm cryopreservation depend on their mass concentration instead of their molar concentration [38]. Monosaccharides can be mixed more easily and efficiently even in concentrated solutions of CPs because they have a lower viscosity than disaccharides [36]. Disaccharides such as sucrose, trehalose [31] and lactose [32] have been used as non-permeant CPs in vitrification solutions, but sucrose and trehalose are commonly used [33]. Raffinose (polysaccharide) has also proven to be effective in increasing the survival rate of embryos after vitrification [33]. Furthermore, the addition of mixture of two sugars (sucrose and glucose) in vitrification medium has more effectively improved the survival rate of the vitrified bovine blastocysts than the addition of sucrose alone [30].

Natural honey is a mixture of 25 sugars (mainly fructose and glucose) accounting for approximately 95 to 97 % of its dry matter. Besides saccharides, a large number of other bioactive substances such as organic acids, enzymes, antioxidants and vitamins are present in honey in traces.[42,43,45]. Such unique composition provides numerous nutritional, biological and pharmacological effects in living cells, i.e. antimicrobial (anti-viral, anti-fungal and anti-bacterial), antioxidant and anti-toxins, anti-inflammatory, anti-mutagenic, anti-cancer and

immunosuppressive activities [42,43,45,47-50]. To the best of our knowledge, natural honey has not been yet studied as a cryoprotectant in cryopreservation media. Since honey is a rich-mixture of sugars, we hypothesize that natural-unheated honey in vitrification medium can induce efficient-cell dehydration in bovine oocytes.

3.3 Materials and methods

3.3.1 Oocyte collection

Bovine ovaries were collected from an abattoir and transported at approximately 25 °C to the laboratory within 7-8 h after collection. Follicles between 2-8 mm in diameter were aspirated. Cumulus-oocyte complexs (COCs) were evaluated in Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen Inc., Burlington, ON, Canada), supplemented with 5% (v/v) new-born calf serum (CS; Invitrogen Inc.). Grade-1 COCs (with uniform cytoplasm and >3 layers of cumulus cells) were selected and washed three times in DPBS + 5% CS in order to be used.

3.3.2 In vitro maturation (IVM)

Selected oocytes were washed three times in maturation media (TCM-199 [Gibco/Invitrogen, 12340] supplemented with 5% CS, 0.5 μg/mL FSH (Bioniche, Belleville, ON, Canada), 5 μg/mL LH (Bioniche) and 50 μg/mL gentamicin). Groups of 18 to 22 COCs were deposited in 100 μL droplets of maturation media (under mineral oil) and incubated for 24 h at 38.5 °C, 5% CO_2, and high humidity in air. After in vitro maturation, COCs were denuded with 0.3% hyaluronidase (Sigma, H3506) in Ca^{2+}-Mg^{2+} free DPBS (Invitrogen Inc.) and only matured oocytes appearing morphologically normal and possessing first polar body with homogeneous ooplasm were selected under a stereomicroscope for the dehydration-rehydration procedures.

3.3.3 Preparation of natural honey media

The honey (floral source includes mainly alfalfa, and combinations of clover, canola, wolf willow, caragana, wild rose and dandelion) used in this study was procured from a local beekeeper (T&H Apiaries, Solliosy's Honey, Saskatoon, SK, Canada, S7M 3W7). First, the osmotic pressure of 10% (w/v) natural-honey solution in de-ionized water was measured ten times using a vapor-pressure osmometer (VAPOR®, model # 5520, Wescor Inc. Logan, Utah, USA). Based on calculations, the osmotic pressure of 21.74% (w/v**)** honey was equivalent to 1000 mOsm (1M). Five concentrations (0.25M , 0.5M, 1M, 1.5M and 2M) of natural honey and sucrose were prepared fresh in TCM-199 supplemented with 5 % CS, filtered through 0.45 mm filter (tissue culture filter) and incubated at 38.5°C under 5% CO_2 in air for at least 3 h before use.

3.3.4 Oocyte imaging during dehydratation-rehydratation procedures

Denuded oocytes were randomly distributed into 11 groups (Figure 3.1), i.e. control (no sugar) and five concentrations of each honey and sucrose (0.25M, 0.5M, 1.0M, 1.5M or 2.0M in TCM-199 + 5% CS). Oocytes were incubated at 38.5°C in a 5% CO2 air and high humidity for at least 3 h before their evaluation during dehydration and rehydration procedures. On each day of experiment, at least two oocytes per treatment group were analyzed for imaging. Mature oocytes were held individually with a glass holding micropipette (ICSI micropipettes, REF: MIC-50-30, HUMAGEN, Jyllinge, Denmark) under Inverted Nikon microscope equipped with micromanipulators (TransferMan NK_2, Eppendrof AG 2231, Hamburg, Germany) and viewed using a Nikon D90 camera attached to a monitor. During this procedure, continuose video recordings were done from the start to the end of the procedure. Each oocyte was first kept in 40

µl droplets of the control media (TCM-199 + 5 % CS) for 1 min, and then transferred into test medium (TCM-199 + 5 % CS + sucrose or honey test solutions) for 3 minutes. After that, the oocytes were transferred into another droplet, as control medium (TCM-199 + 5 % CS) for 3 min for rehydration (Figure 3.2). During dehydration or rehydration procedures, the images were captured from recorded videos at intervals of 0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 150, 180 sec using VideoMach 5.8.3 software (http://www.gromada.com/) Each single oocyte has 24 images (see appendix), and each image was overlaid to evaluate the volumetric changes in oocyte during dehydration and rehydration phases.

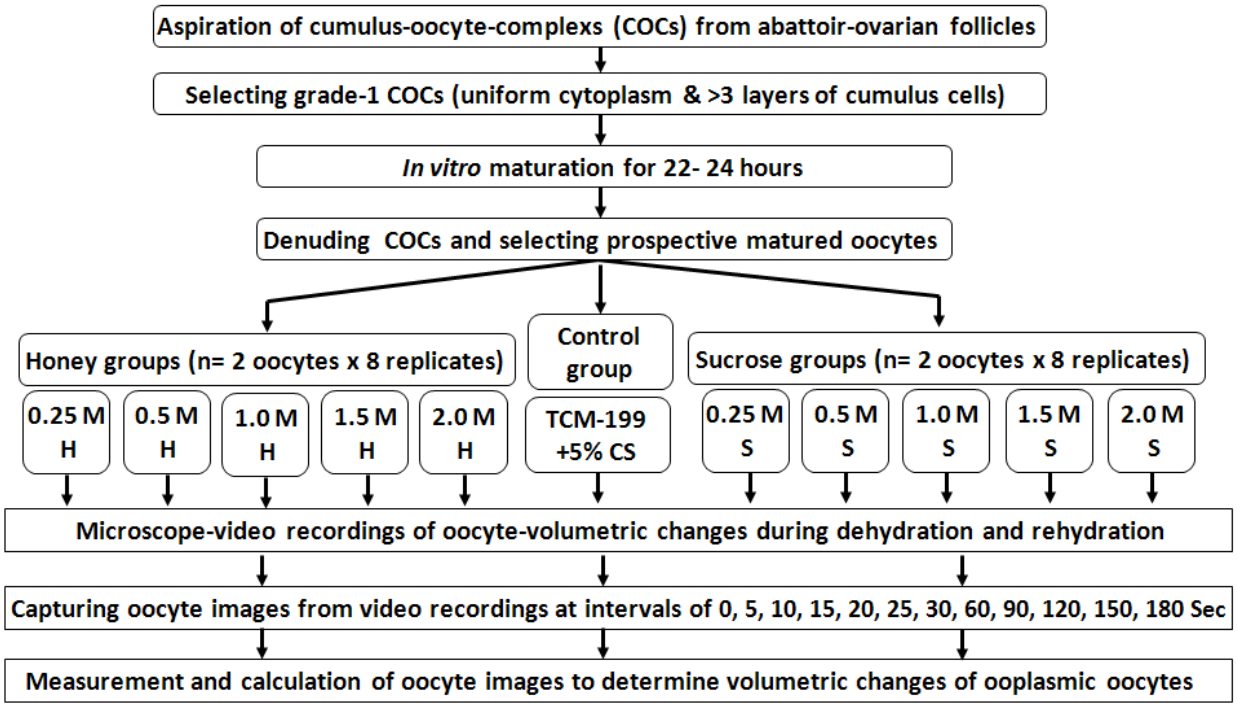

Figure 3.1 Experimental design for the oocyte dehydration in different concentrations of honey and sucrose.

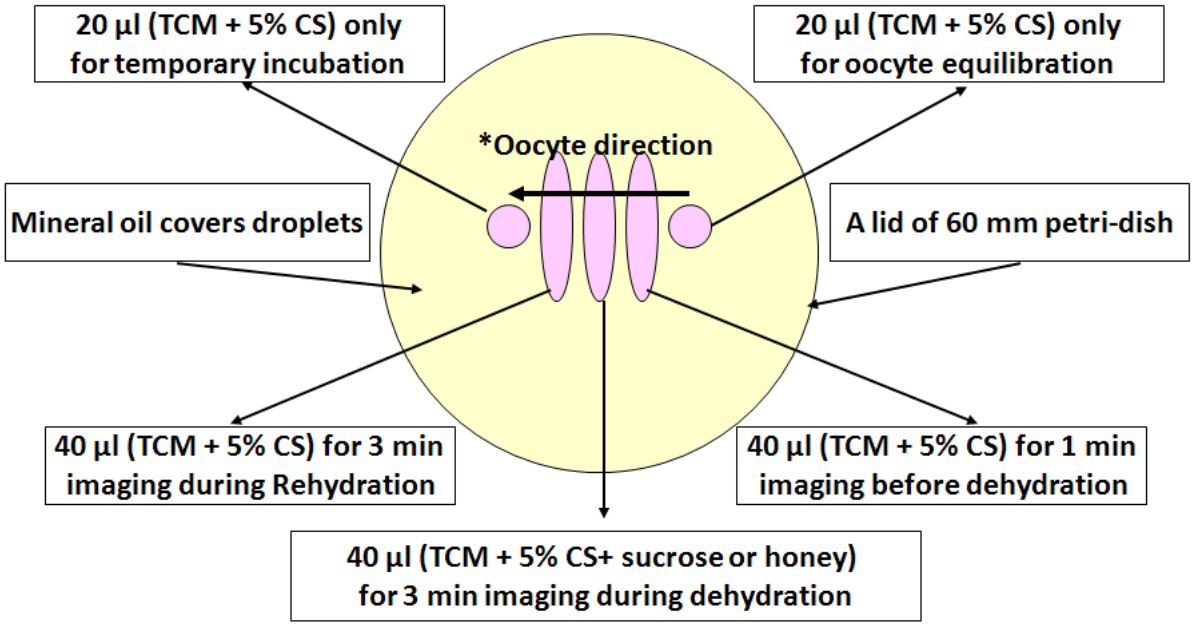

Figure 3.2 Schematic diagram of microscope-video imaging of oocytes during dehydration and rehydration phases.

3.3.5 Measurements and calculations

Image-J ver.1.42 software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) was used to measure the minimum and maximum radii of ooplasm of oocytes. The volume of oocyte's ooplasm was calculated using following formula assuming oocyte as a spheroid:

$$\text{Ooplasm volume} = 4/3 * \pi * r1 * r2 * r3$$

where r1 and r2 are maximum and minimum radii, and r3 was assumed to be equal to the minimum radius.

3.3.6 Statistic analysis

Data collected from eight replicates were analyzed using SAS® Enterprise Guide 4.2 (SAS, Cary, NC, USA). The statistical analysis included following: (a) Proc Mixed repeated-measures one-way analysis of variance (time as independent discrete variable with randomized complete block design) in order to find out the time point by which the dehydration or rehydration is completed (i.e., equilibrium is obtained) in each concentrations of honey and sucrose-based medium. (b) Proc Mixed repeated-measures factorial (compound*concentration*time) analysis of variance (time as independent discrete variable) with randomized complete block design was carried out in order to determine the effect of CP (honey and sucrose), concentrations (0.25M, 0.5M, 1M, 1.5M and 2M), time (0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 150 and 180 sec) and their interactions on ooplasm volume during dehydration and rehydration. Polynomial regression was calculated to determine the relationship between time (x) and ooplasm volume (y) during dehydration and rehydration phases for each concentration of honey and sucrose.

3.4 Results

Volume of bovine oocytes decreased significantly to 68%, 55% and 47 % of iso-osmotic volume after their exposure to hyperosmotic media containing 0.25M, 0.5M and 1.0M natural honey within 60 sec; whereas maximum shrinkage reached to 38% and 41% within 30 and 20 sec in higher concentrations of 1.5M and 2.0M, respectively (Figure 3.3 and Table 3.2). In addition, the ooplasm of oocytes regained their original volume and fully rehydrated in the isotonic medium (control) within 60 to 90 sec (Figure 3.3).

During dehydration of bovine ooplasm (oocyte) in 0.25M and 0.5M sucrose-based media, oocyte volume decreased to 72% and 54% within 60 sec, respectively. In 1M, 1.5 and 2 M concentrations of sucrose-based media, the maximum dehydration of ooplasm (oocyte) occurred within ≤30 sec (Figure 3.4). During rehydration, oocytes dehydrated in 0.25M, 0.5M and 1M sucrose-based medium reached to almost original volume within 90 to 120 sec, but oocytes dehydrated in 1.5M or 2M rehydrated within 10 and 20 sec respectively (Figure 3.4).

In addition, no significant difference in oocyte-volume changes were observed during dehydration ($p=0.990$) and rehydration ($p=0.485$) phases, due to CPs (honey vs. sucrose). Effects of five concentrations of honey and sucrose based medium on volume changes were significantly different during dehydration ($p=0.0002$) and rehydtration ($p=0.0285$) phases (Figure 3.5). In other words, rate of oocyte dehydration and rehydration increases as the concentration of honey or sucrose is increased (figure 3.5, Table 3.1).

The relationships between time and ooplasm volume during dehydration and rehydration phases in different concentrations of sucrose and honey are presented in Table 3.1. The correlation coefficients between time and concentrations during dehydration and rehydration phases were significant ($P<0.01$) in all concentrations of both sucrose and honey. However, the correlation coefficients between between time and ooplasm volume during dehydration and rehydration in control group were not significant ($P>0.05$). Polynomial regression analysis revealed that ooplasm volume changed with time following cubic regression.

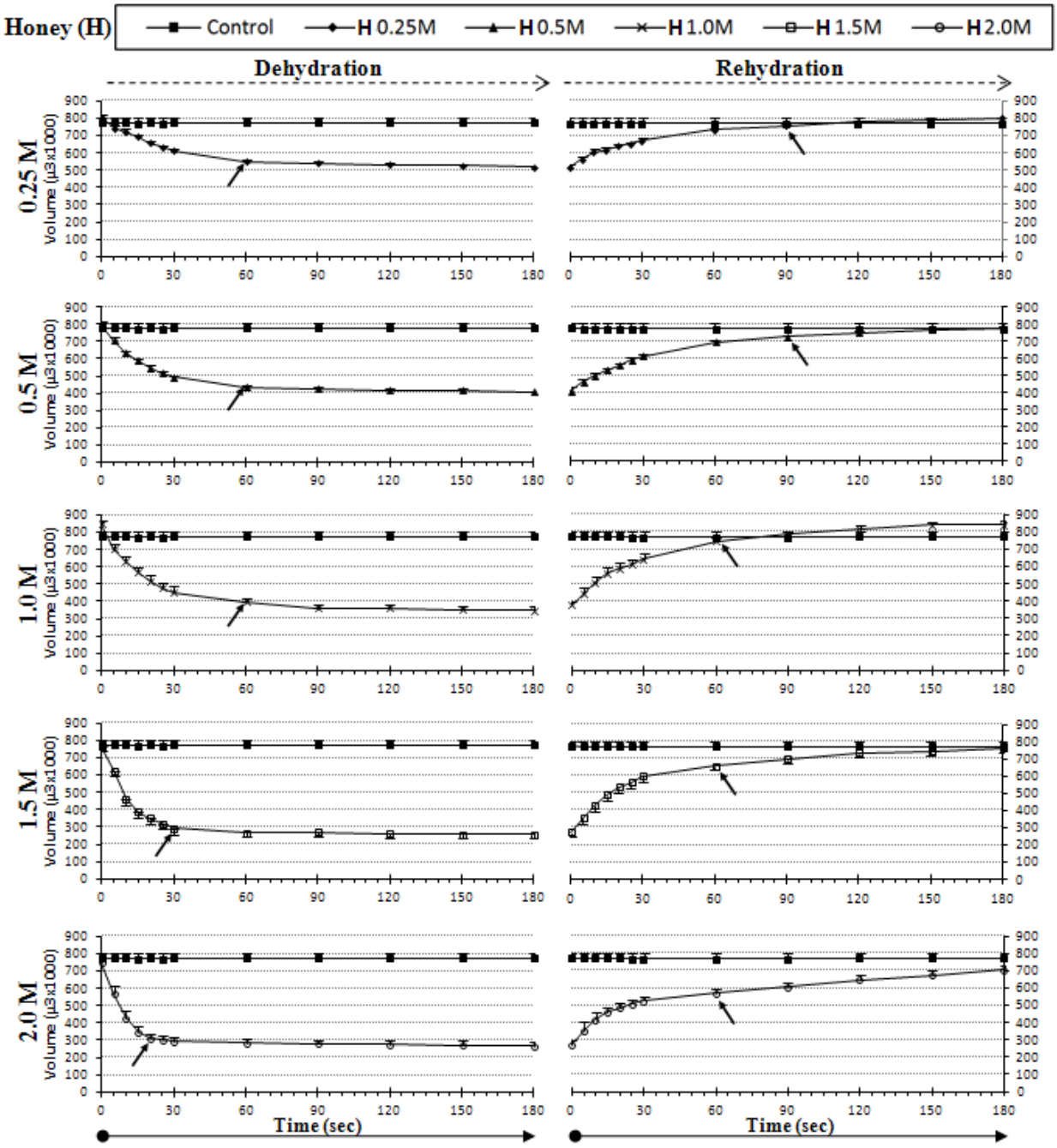

Figure 3.3 Volumetric changes in bovine ooplasm oocytes as a function of time during dehydration (left panels) in different concentrations of natural honey-based media, and rehydtration in TCM (right panels). The ooplasm volume was calculated from maximum and minimum radii assuming oocyte has a spheroid shape. The volume was calculated at 0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 150 and 180 sec after exposure to dehydration and rehydration media. Each point represents a mean±SEM of 8 oocytes. Arrows indicate the time point when the maximum dehydration and rehydration occurred in ooplasm and its volume did not change statistically at subsequent time points.

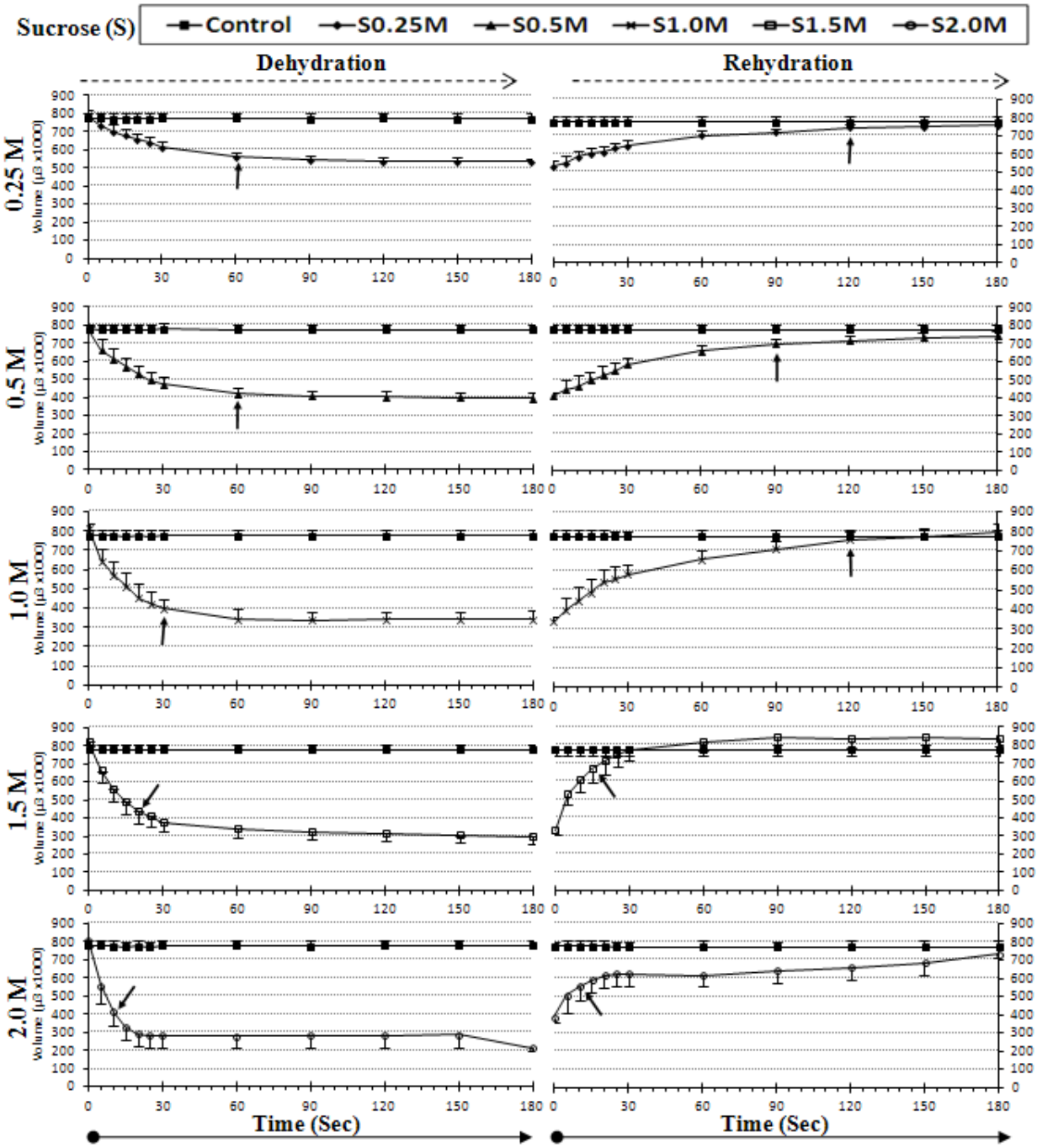

Figure 3.4 Volumetric changes in bovine ooplasm (oocytes) as a function of time during dehydration (left panels) in different concentrations of sucrose-based media, and rehydtration in TCM (right panels). The ooplasm volume was calculated from maximum and minimum radii assuming oocyte a spheroid shape. The volume was calculated at 0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 150 and 180 sec after exposure to dehydration and rehydration media. Each point represents a mean±SEM of 8 oocytes. Arrows indicate the time point when the maximum dehydration and rehydration occurred in ooplasm and its volume did not change statistically at subsequent time points.

Table 3.1 Relationship between time (x) and ooplasm volume (y), during dehydration and rehydration phases, in different concentrations of sucrose and honey.

Phase	CP	Conc.	Polynomial regression	Correlation coefficient
Dehydration	Control	0	$y= 0.0000x^2 – 0.0051x +764.8$	r = 0.104, P>0.05
	Sucrose	0.25 M	$y= -0.0002x^3 + 0.0553x^2 - 6.3255x +768.38$	r = 0.995, P<0.01
	Honey	0.25 M	$y= -0.0002x^3 + 0.0687x^2 - 7.5136x + 789.79$	r= 0.995, P<0.01
	Sucrose	0.5 M	$y= -0.0003x^3 + 0.1088x^2 - 10.951x +723.66$	r= 0.980, P<0.01
	Honey	0.5 M	$y= -0.0003x^3 + 0.1128x^2 - 11.365x + 751.48$	r= 0.982, P<0.01
	Sucrose	1.0 M	$y= -0.0005x^3 + 0.1532x^2 - 14.842x + 730.65$	r= 0.962, P<0.01
	Honey	1.0 M	$y= -0.0004x^3 + 0.1396x^2 - 14.141x + 777.82$	r= 0.975, P<0.01
	Sucrose	1.5 M	$y= -0.0005x^3 + 0.1611x^2 - 15.481x + 730.95$	r= 0.956, P<0.01
	Honey	1.5 M	$y= -0.0006x^3 + 0.1839x^2 - 16.999x + 669.17$	r= 0.934, P<0.01
	Sucrose	2.0 M	$y= -0.0007x^3 + 0.2135x^2 - 18.139x + 643.46$	r= 0.886, P<0.01
	Honey	2.0 M	$y= -0.0006x^3 + 0.178x^2 - 15.868x + 624.84$	r= 0.899, P<0.01
Rehydration	Control	0	$y= 0.0001x^2 – 0.0238x + 773.87$	r= 0.545, P>0.05
	Sucrose	0.25 M	$y= 9E-05\ x^3 - 0.034x^2 - 4.5102x +534.21$	r= 0.998, P<0.01
	Honey	0.25 M	$y= 0.0001x^3 - 0.0468x^2 + 5.788x + 535.72$	r= 0.995, P<0.01
	Sucrose	0.5 M	$y= 0.0001x^3 - 0.0515x^2 + 6.8273x +405.66$	r= 0.998, P<0.01
	Honey	0.5 M	$y= 0.0002x^3 - 0.0633x^2 + 7.8479x + 423.8$	r= 0.997, P<0.01
	Sucrose	1.0 M	$y= 0.0002x^3 - 0.08x^2 + 9.9101x + 327.58$	r= 0.988, P<0.01
	Honey	1.0 M	$y= 0.0002x^3 - 0.0816x^2 + 10.024x + 406.8$	r= 0.994, P<0.01
	Sucrose	1.5 M	$y= 0.0005x^3 - 0.1568x^2 + 15.173x + 433.7$	r= 0.952, P<0.01
	Honey	1.5 M	$y= 0.0003x^3 - 0.1091x^2 + 11.863x + 310.27$	r= 0.986, P<0.01
	Sucrose	2.0 M	$y= 0.0003x^3 - 0.0871x^2 + 7.6963x + 459.36$	r= 0.915, P<0.01
	Honey	2.0 M	$y= 0.0003x^3 - 0.0837x^2 + 8.946x + 318.44$	r= 0.980, P<0.01

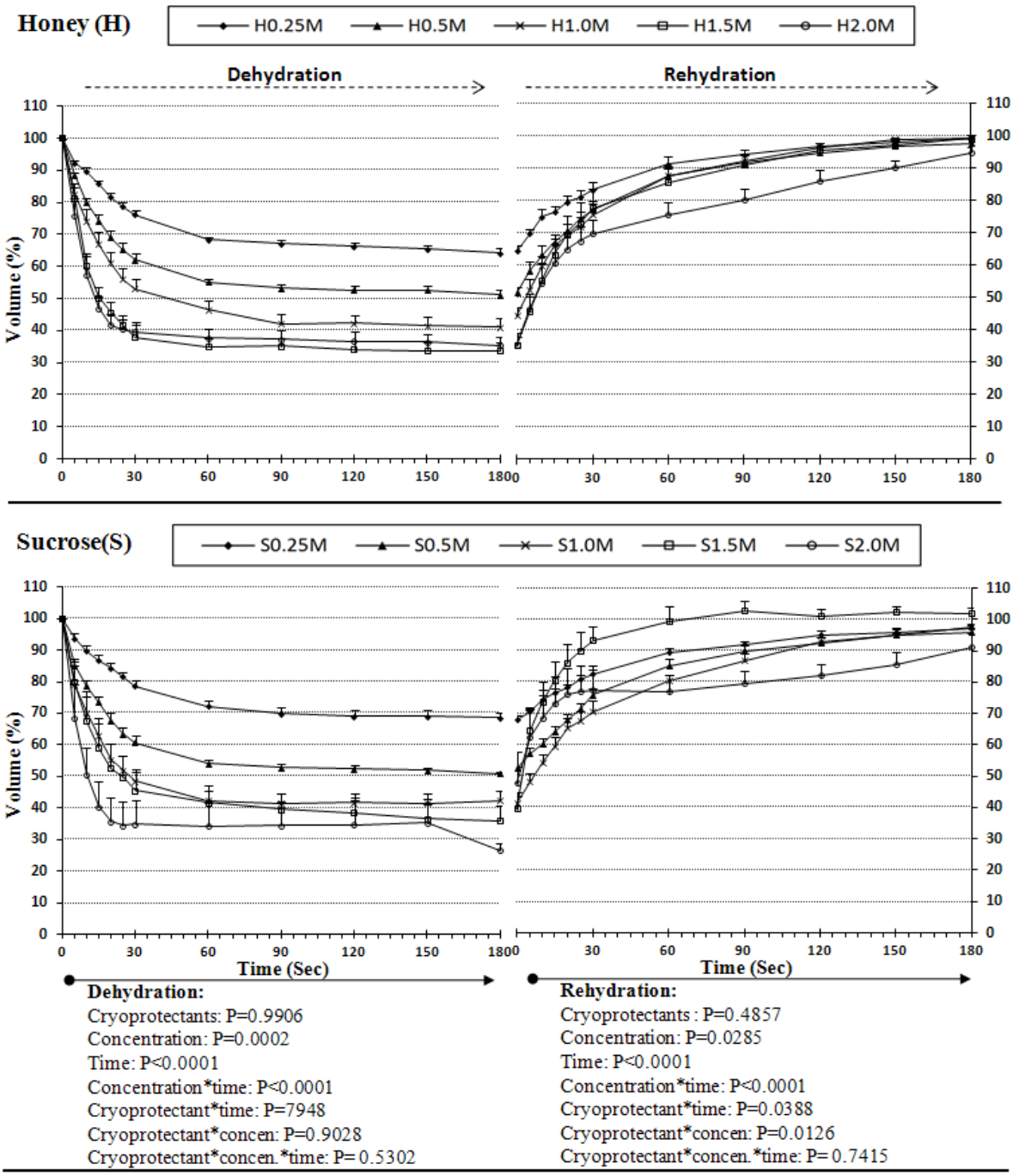

Figure 3.5 Effect of CPs (honey vs. sucrose), their concentrations (0.25M, 0.5M, 1M, 1.5 and 2M), time (0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 150 and 180 sec) and their interactions on percent change in ooplasm (oocyte) volume (initial volume=100%) during dehydration (left panels) and rehydration (right panels) phases. Each point represents a mean±SEM of 8 oocytes.

Table 3.2 Exposure time required to cause maximum change in oocyte volume during dehydration in different concentrations of honey- vs. sucrose- based media. (Mean±SEM; N=8 oocytes).

Concen. (Mol/L)	Honey-based medium			Sucrose-based medium		
	Time (sec)	Volume*1000μ^3	Volume (%)	Time (sec)	Volume*1000μ^3	Volume (%)
0.25 M	**60**	**550 ±8**	**68.3 ±1.0**	**60**	**564 ±25**	**72.1 ±1.8**
0.50 M	**60**	**436 ±12**	**55.1 ±1.2**	**60**	**419 ±24**	**54.2 ±1.1**
1.00 M	**60**	**398 ±3**	**46.5 ±2.9**	**30**	**397 ±39**	**48.6 ±3.9**
1.50 M	**30**	**291 ±33**	**37.9 ±3.8**	**20**	**438 ±72**	**52.6 ±7.9**
2.00 M	**20**	**309 ±24**	**41.8 ±3.5**	**10**	**409 ±76**	**50.6 ±8.7**

3.5 Discussion

Our hypothesis in this study was that natural honey-based media can cause the similar dehydration of bovine oocytes similar to that caused by sucrose-based media. Our study demonstrated that honey and sucrose-based medium have a similar effect to the volumetric changes on *in vitro* matured bovine oocyte (Figure 3.5). As expected, the volumetric change is dependent on the concentration of the honey or sucrose; the ooplasm-oocyte volume decreased when the concentration of honey and sucrose-based media increased. To the best of our knowledge, honey has not yet been studied as a cryoprotectant agent to cause cell dehydration. The similarity between honey and sucrose in cell dehydration was based on the fact that natural honey is a mixture of about 25 saccharides (mainly fructose and glucose), which account for approximately 95% dry matter [42]. Fructose and glucose have proven to serve as good osmotic buffers in cryopreservation media because they have a lower viscosity than disaccharides [36]. Based on our results, we propose that natural honey will be a suitable alternative to sucrose in cryoprotectant media.

Criteria for selecting appropriate concentrations of sucrose and honey to be used in cryo-protective media should be based on the volume at which oocytes can obtain maximum dehydration without causing any physical damage to the cell. The criteria should also consider the fact that intracellular water consists of free water and bound water. One lethal issue for the living cells is the loss of the bound water because water molecules form "hydration shell" around various cellular molecules such as proteins, DNA, RNA and membrane phospholipids, and thus protect their structure and functions [41]. Therefore, during dehydration of oocytes, a delicate balance must be maintained between drawing out free water (tends to form ice crystals rapidly) while not disturbing the bound water in order to achieve a safe (unharmed) dehydration of the intracellular compartment. Otherwise, post-warming cell viability will be diminished as a result of losing structural support to the intracellular proteins and lipids. According to the previous studies, the osmotically inactive volume (i.e. proportion of cell volume that has no response to extracellular hyperosmotic pressure) of MII bovine oocytes in the presence of NaCl [115] or sucrose [199] were determined to be 24.7% and 26.1% of the isotonic cell volume, respectively. This means that volume of intracellular-free water in a mature bovine oocyte averages 75% of the original volume. A study has reported that the osmotically inactive volume of a mature human oocyte is about 19%, indicating that volume of intracellular-free water is approximately 80% of the oocyte volume [40]. Another study has found that osmotic-tolerance limit is about 57% (of the original volume) to dehydrate human oocytes safely using the concentration of 0.4M sucrose [200]. Our study shows that bovine oocytes in the concentration of 0.5M sucrose- and honey-based medium require 60 seconds to reach 54% and 55% of the isotonic cell volume respectively (Table 3.2). Increasing the extracellular concentrations of non-permeating cryoprotectants in medium can significantly reduce the required concentrations of permeating

CPs needed for intracellular vitrification [28]. Therefore, an ideal vitrification solution should have a maximum sugar concentration in order to enhance cell dehydration, thus minimizing the quantity of intracellular permeant CPs while not exceeding the osmotic-tolerance limit of oocytes [28]. In this way, sugar alleviates high concentrations of permeating cryoprotectants, and thus decreases their toxicity [40,41]. Our study found that if sucrose-based medium increased from 0.5M (commonly used concentration) to 1M (or higher), oocyte dehydration improved from 54% (of isotonic volume) at 60 sec to 49% at 30 sec (Table 3.2). To achieve the maximum and safe dehydration prior to the oocyte vitrification, the concentration of 1M sucrose (or higher) should be avoided because this level of sucrose concentration cause a faster dehydration (≤30sec) and mechanical damage to the oocytes (Figure 3.4 & table 3.2). Based on our dehydration study, we suggest the use of 1M honey-based medium for oocyte vitrification. The rationale is that the oocytes reached maximum dehydration (46%) and regained original volume at 60 second each in medium containing 1M honey. Also, this concentration demonstrated a superior dehydration ability than 0.5 M sucrose-based media to withdraw ~10% more of intracellular active water (as mentioned above that volume of intracellular-free water averages 75% of the original volume of bovine oocyte). Therefore, 1M (21.7%w/v) natural honey-based medium appeared to be suitable as a non-permeant CP in vitrification medium in order to obtain sufficient and safe dehydration during vitrification procedure of bovine oocytes. Further study was conducted to investigate the effect of different concentrations of natural honey as non-permeant CP in vitrification medium on post-warming maturation, cleavage and embryonic development of vitrified bovine oocytes.

In polynomial regression analysis, a lack of relationship between time and ooplasm volume change in control group clearly demonstrate the validity of our procedure to measure the ooplasm volume. However, the ooplasm volume changed over time in all concentrations of

sucrose and honey following curvilinear pattern (3^{rd} degree cubic regression). Generally, the ooplasm volume decreased (in case of dehydration) and increased (in case of rehydration) linearly during first 30-60 sec and then became stable afterwards.

In conclusion, two main factors limiting the success of post-warming viability of vitrified oocytes are the formation of intracellular ice crystals and the cytotoxicity associated with high concentration of CPs. Our study focused on improving post-warming viability of vitrified oocytes by ensuring sufficient dehydration of cells prior to vitrification using natural honey. Natural honey has similar dehydration effect on bovine oocytes as sucrose, the most commonly used sugar in vitrification procedure. Natural honey (1M, or 21.7% w/v) for 60 seconds was found to be a suitable concentration (combination) to dehydrate bovine oocytes, and it can be used in vitrification medium.

Acknowledgments:

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CHAPTER 4: VITRIFICATION OF IMMATURE BOVINE CUMULUS-OOCYTE-COMPLEXES USING NATURAL HONEY AS A NON-PERMEANT CRYOPROTECTANT

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4.1 Abstract

The main objective of this study was to determine the property of honey as a nonpermeant cryoprotectant in vitrification of bovine oocytes. Two experiments were conducted to achieve this objective. The first experiment was conducted to validate the optimal concentration of a honey-based medium for vitrification of bovine cumulus-oocyte-complexs (COCs) on the basis of in vitro maturation as an endpoint assessment. The COCs were randomly distributed into: Control (G1, not vitrified), sucrose vitrified (0.5M, G2) and honey vitrified (0.5M, 1M, 1.5M designated as G3, G4 and G5 respectively) groups. The COCs were first equilibrated in vitrification solution 1 (VS1; TCM-199 + 7.5% DMSO + 7.5% EG + 20% calf serum) at ~ 22^{o}C for 5 min and then exposed to vitrification solution 2 (VS2; TCM-199 + 15% DMSO + 15% EG + 20% calf serum + 17.1% sucrose or respective honey concentrations) for 1 min, mounted on Cryotops® and plunged into LN_2. The COCs were warmed (in TCM-199 + 20% calf serum + honey or sucrose) at 38.5^{o}C for 1 min, washed, and underwent in vitro maturation (IVM), for 24 hrs, denuded, and evaluated for maturation status with lamin-AC/DAPI immunostaining. Results revealed that maturation rate was higher in non-vitrified control group (G1; 80.7%) than in vitrified groups (56%, 52%, 55% and 51% in G2, G3, G4 and G5 groups, respectively) (P=0.0003), whereas there was no significant difference in maturation rate among vitrified groups (P>0.05). A second experiment was conducted to evaluate in vitro fertilization (IVF) and embryonic development of oocytes vitrified in medium containing 1.0M honey or

0.5M sucrose. Selected COCs were distributed as control (not vitrified, NV) and vitrified either in 1.0M honey (HV) or 0.5M sucrose (SV), and underwent IVM, IVF and IVC for 9 days. Data revealed that cleavage rate was higher in the control group (74%, NV, n=183) than in vitrified groups (51% in HV, n=137; and 42% in SV, n=131; P<.0001); without any significant difference (P=0.0723) among vitrified groups (HV and SV). Rate of blastocyst formation was higher (P<.0001) in NV (34%) than in HV and SV groups. Blastocyst formation rate in the HV group was higher than in the SV group (13% vs. 3% respectively; P=0.0026). In conclusion**,** natural honey (1.0M or 21.7%w/v) in vitrification medium has superior effect over sucrose (0.5M) to improve post-warming embryonic development of vitrified bovine oocytes.

Key words: bovine oocytes, vitrification medium, natural honey, sucrose and viability.

4.2 Introduction

The most common cryopreservation method of genetic preservation used for domestic and wild animals is semen freezing. Preservation of female genetics is important to ensure complete protection of animal genetics. Such preservation of female germplasm can be done through the cryopreservation of ovarian-cortex tissue, oocytes and embryos, in order to establish a germplasm bank that can serve as a tool for enhancement and maintenance of animal breeding programs and assisted reproduction in humans [1-3]. There are several barriers limiting successful cryproservation of mammalian oocytes including large size of the oocyte, low surface area to volume ratio, high intracellular water content and low hydraulic conductivity [6]. Besides these factors, the cellular morphology and molecular sensitivity to chilling present an additional difficulty for successful oocyte cryopreservation [7-10]. Successful vitrification in embryos has been reported in many species including mouse [137], sheep [138], cattle [139], horse [140], rabbit [14] buffalo [141] and human [142]. However, vitrifcation of oocytes has been attempted

in many species including mouse [11], cattle [12], buffalo [13] and human [15], but with very limited viability after warming [7,201]. Inspite of the encouraging results from vitrified matured bovine oocytes [16-18,21], numerous studies have reported an extremely low rate of blastocyst formation [7,19-21], indicating the need for optimization of vitrification procedure.

The optimal vitrification procedure needs to reduce the effects of several damaging factors including osmotic stress, CP toxicity and ice crystallization to the cells/tissue to be vitrified [18,22]. Sugar incorporation in the vitrification medium is one of the many attempts for optimization of procedure, and it is aimed to minimize the effect of such factors assumed to limit the success of post-warm cell viability [24]. It has been reported that including sugar in vitrification solution could significantly improve the survival rate of vitrified bovine blastocysts and human-immature oocytes [29,30]. The role of sugar in the vitrification media is to cause an hyperosmotic gradient across the cell membrane which results in the withdrawal of intracellular active water and thus reduces the chances of intracellular ice formation, and minimizes lethal freezing injury [25,26]. In addition, sugar enhances viscosity of the intracellular solutes minimizing intracellular-toxic effects of the permeating cryoprotectants (CPs) [27,28].

Fructose and glucose not not exibit any toxic effects when used in sperm cryopreservation [34,35]. Monosaccharides appeared to be more effective for cryproservation of sperm than disaccharides as shown with studies on deer sperm [146] and dog sperm [147]. . In addition, monosaccharides have a higher tendency to dissolve and mix efficiently with concentrated CP solutions, and their density is lower than that of disaccharides [36]. Fructose has a better effect on semen quality than disaccharides and polysaccharides [146]. For instance, in dog sperm cryopreservation, sucrose and trehalose were found to improve sperm viability and reduce damage, but without promoting post-thaw motility [147] while using fructose improved

both the intact acrosome and motility. Furthermore, the effect of different sugars on mouse sperm cryopreservation depends on their mass concentration rather than molar concentration [38]. The addition of disaccharides in vitrification media such as sucrose, trehalose[31] and lactose [32] have been used as non-permeant CPs with sucrose being the most commonly used [33]. Polysaccharides such as raffinose have also proven to be effective in improving the viability rate and subsequent embryonic development after cryopreservation [33,37]. Moreover, the addition of a mixture of sucrose and glucose in the vitrification medium has more effectively improved the viability rate of the post-warming bovine blastocysts than the addition of sucrose alone [30]. In our previous study (chapter 3), honey-based vitrification medium has proven to have same dehydration efficiency as sucrose. Honey consists of a rich mixture of about 25 different sugars, mainly fructose and glucose. Excluding the water in honey (about 17%), the sugars in honey account for 95 to 97 percent of the dry matter [42-44]. As well, in honey there are at least 181 other minor bioactive substances that are mostly present in trace amounts [44]. This complex mixture of sugars, organic acids, enzymes, vitamins and minerals provides numerous nutritional, biological and pharmacological effects in living cells. These beneficial effects include anti-inflammatory, anti-mutagenic, anti-cancer, anti-microbial, antioxidant and anti-cytotoxicity properties [42,43,48-52]. It was suggested from our previous dehydration study (chapter 3) that optimal concentration of honey (1M, or 21.7% w/v) can be used in vitrification medium in order to improve post-warming viability of vitrified COCs. We expected improvement in the viability of vitrified COCs when safe and sufficient oocyte dehydration occurs prior to vitrification using (1M, or 21.7%) honey. Therefore, in the present study, we investigated the effect of natural honey as a non-permeant CP in the vitrification medium on post-warming viability of bovine COCs and subsequent embryonic development.

4.3. Materials and methods

4.3.1 Chemicals and supplies

Cryotops® were purchased from Kitazato Biopharma Supply Co. (Fujinomiya, Japan). All chemicals in this study were purchased from Sigma (St. Louise, MO, USA), unless otherwise indicated. The natural honey used in this study was procured from a local beekeeper (T&H Apiaries, Solliosy's Honey, Saskatoon, SK, Canada, S7M 3W7). The floral source of the honey includes mainly alfalfa, and combinations of clover, canola, wolf willow, caragana, wild rose and dandelion.

4.3.2 Oocyte collection

Bovine ovaries were obtained from an abattoir and transported to the laboratory at approximately 25 °C within 7-8 h after collection. Cumulus-oocyte-complexs were aspirated from follicles between 2-8 mm in diameter.The COCs were screened under a stereomicroscope and washed three times in Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen Inc., Burlington, ON, Canada), supplemented with 5% newborn calf serum (v/v; CS; Invitrogen Inc.). Grade-1 COCs were selected based on their morphological appearance (uniformly granulated cytoplasm surrounded by more than 3 layers of compact cumulus cells) for further experimentation.

4.3.3 Preparation of natural honey media

First, the osmotic pressure of 10% (w/v) natural-honey solution was measured ten times by using a vapor-pressure osmometer (VAPOR®, model # 5520, Wescor Inc. Logan, Utah, USA). Based on calculations, the osmotic pressure of 21.74% (w/v**)** honey was equal to 1000 mOsm which is equivalent to 1M. Three concentrations (0.5M, 1M and 1.5M) of natural honey

vitrification medium were prepared as final vitrification solution (VS2) and filtered to be used fresh.

4.3.4 Vitrification and warming procedures

Vitrification of COCs was performed using Cryotop® method [15] with some modifications. All COCs were first equilibrated in vitrification solution 1 (VS1; TCM-199 + 7.5% v/v dimethyl sulfoxide (DMSO) + 7.5% v/v ethylene glycol (EG, v/v) + 20% v/v calf serum (CS, v/v)) for 5 min at room temperature (22 to 25 °C). After equilibration, COCs were transferred through three 30 µL droplets of vitrification solution 2 (VS2) that contained either natural honey (0.5M, 1M and 1.5M) or sucrose (0.5M) in TCM-199 + 15% v/v EG + 15% v/v DMSO + 20% v/v CS at 37 °C within 1 min. Then, 3-5 COCs were loaded onto the filmstrip of a cryotop (Kitazato Biopharma Supply Co.; Fujinomiya, Japan) and immediately plunged into LN_2. Prior to vitrification, the surrounding solution was removed by gentle aspiration, leaving a thin layer around the oocytes. For warming of COCs, the cryotop was immersed in 38.5 °C warming solution (WS) consisting of TCM-199 + 20% v/v CS supplemented with desired concentration of natural honey (0.5, 1.0, 1.5M) or sucrose (0.5M) for 1 min. After warming, COCs were washed three times (3 to 5 min each) in TCM-199 + 5% CS.

4.3.5 In vitro maturation (IVM)

The vitrified-thawed COCs were washed three times in maturation media (TCM-199 supplemented with 5% CS, 5 µg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 µg/mL FSH (Bioniche) and 0.05 µg/mL gentamicin). COCs (n=18-20) were placed in 100 µL IVM-media droplets under mineral oil, and incubated for 22 to 24 h at 38.5 °C, 5% CO_2 in air and high humidity.

4.3.6 Oocyte immunostaining (Lamin-AC/DAPI Staining)

This immunostaining protocol (Lamin-AC/DAPI Staining) was developed in our labrotory for an accurate evaluation of nuclear maturation of immature bovine oocytes. After in vitro maturation, vitrified-warmed COCs were completely denuded (by pipetting 80 to 100 times avoiding bubble formation) in 0.3% hyaluronidase in Ca^{2+}/Mg^{2+} free DPBS (Invitrogen Inc.). The denuded oocytes were fixed in 4% paraformaldehyde (w/v) in DPBS for 15 min. All subsequent steps were performed at room temperature (RT) and oocytes were washed in DPBS after each step (5 min, 3 times). Oocytes were permeabilized with 0.5% v/v Triton X-100 in DPBS for 30 min followed by additional 30 min of permeabilization in 0.05% v/v Tween-20 (BIO-RAD, Hercules, CA, USA) in DBPS. Oocytes were then transferred in a blocking buffer (2% BSA in DPBS) for 60 min at RT or overnight at 4 °C. After blocking, oocytes were incubated with mouse anti-lamin-AC (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in DPBS + 2% BSA for 60 min followed by washing (3x, 5 min each) and incubation in secondary antibody Alexa 488 labelled anti-mouse IgG (Santa Cruz Biotechnology) in DPBS (1:200) supplemented with 2% BSA, for 60 min. Oocytes were washed (3x, 5 min each) and transferred through at least 3 droplets (5 µl each) of Vectashield Mounting Medium containing 1.5µg/ml of DAPI (Vector Laboratories Inc., Burlingame, CA 94010 USA). Oocytes were mounted on a microscope-glass slide under coverslip supported with paraffin-vaseline (1:1) drops at each corner of the coverslip to avoid oocyte rupture. Finally, oocytes were evaluated for nuclear maturation and nuclear status was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII) (Figure 4.3). Only oocytes reaching metaphase II (MII) stage were considered fully mature.

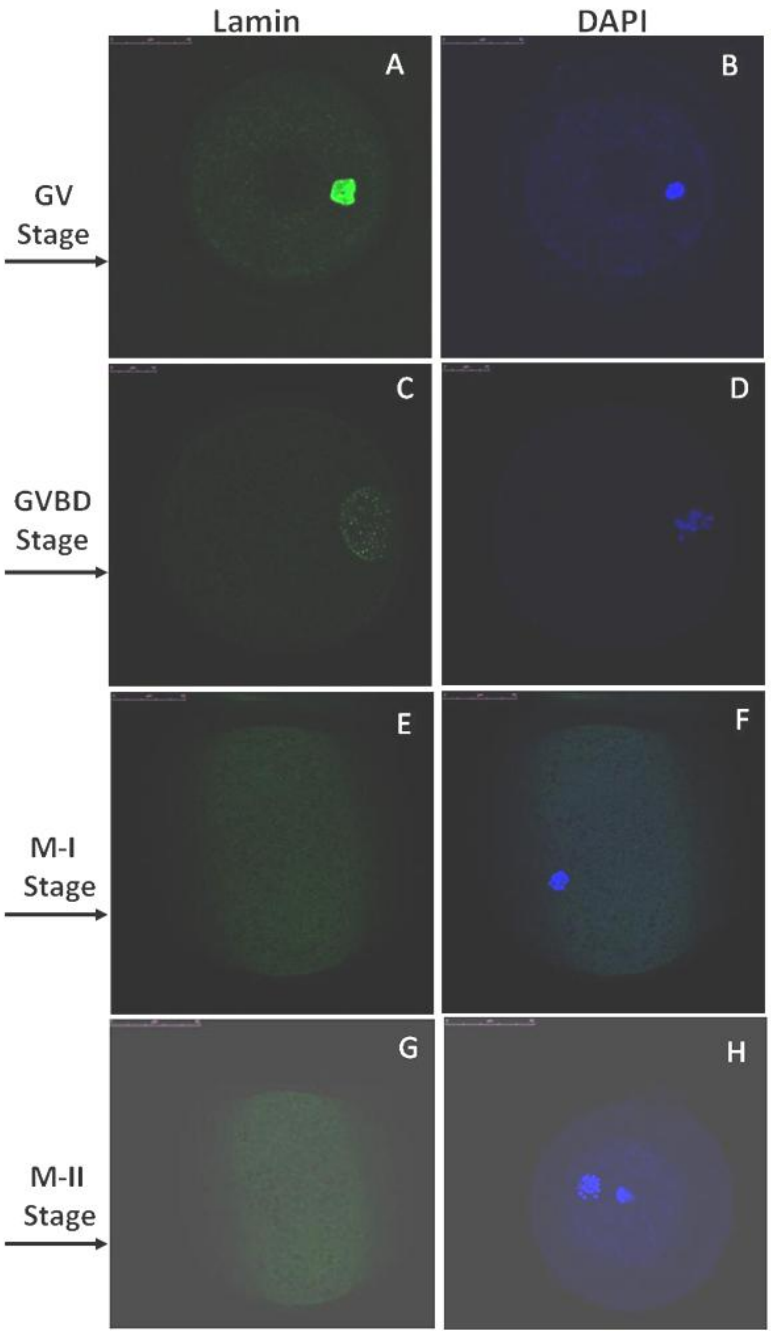

Figure 4.1 Classification of nuclear maturation stages of bovine oocytes after Lamin-AC/DAPI imunostaining. Left panels represent lamin-A/C staining (green) and right panels represent DAPI staining (blue). Bovine oocyte at the germinal vesicle (GV) stage (A) characterized by nuclear envelope and condensed chromatin. Germinal vesicle breakdown (GVBD) stage (B) characterized by observing that the nuclear envelope became faded (C) and outspread chromatin (D). Bovine oocyte at metaphase I stage (MI) has no nuclear envelope (E) with chromatin positioned at the metaphase plate (F). Bovine oocyte at metaphase II stage (MII) has no nuclear envelope (G) with presence of DNA and the first polar body (H).

4.3.7 In vitro fertilization (IVF) and culture (IVC)

Frozen semen from one ejaculate of a single bull was used for all treatments and replicates in this study. Thawed semen was washed through Percoll gradient (45% and 90%) [202] and diluted in Brackett-Oliphant (BO) fertilization media [203] to a concentration of 3×10^6 /mL. Following IVM, groups of 18 to 22 COCs were washed three times in BO supplemented with 10% BSA and then placed into 100 µL droplets of sperm in BO under mineral oil for co-incubation for 18 h at 38.5 °C, 5% CO2 in air and high humidity. The presumptive zygotes were denuded and washed three times through in vitro culture (IVC) medium, i.e CR1aa medium with 5% v/v CS, 1% v/v MEM non-essential amino acids, 2% v/v BME essential amino acids (Invitrogen Inc.), 1% v/v L-Glutamic acid, 0.3% w/v BSA and 0.05 µg/mL gentamicin). Finally, the presumptive zygotes were transferred (18 to 22 zygotes) into 100 µL droplets of IVC-medium under mineral oil and incubated at 38.5 °C under 5% CO_2, 90% N_2, 5% O_2 and high humidity. During *in vitro* culture, cleavage rate was determined after 2 days of *in vitro* fertilization (day 0), whereas, the blastocyst formation was monitored and evaluated on day 9. Control and vitrified COCs in this study were in vitro fertilized and cultured using the same procedures and conditions.

4.3.8 Experimental design

4.3.8.1 Experiment 1:

This experiment was designed to determine the post-thaw maturation ability of immature-bovine COCs vitrified in a medium containing natural honey or sucrose as a cryoprotectants in vitrification medium. An outline of this experiment is illustrated (figure 4.1). In short, after

collection and washing, COCs were distributed randomly into five groups as follow: G1, control (no vitrification); G2**,** 0.5M sucrose; G3**,** 0.5M honey; G4, 1.0 M honey; G5**,** 1.5M honey. Then, COCs were vitrified (as described above in section 4.3.4). After vitrification procedure, oocytes were warmed and *in vitro* cultured for 24h (section 4.3.5). After incubation, vitrified-warmed COCs were completely denuded and immunostained to evaluated for nuclear maturation using Lamin-AC/DAPI (section 4.3.6). In this experiment, three replicates were performed.

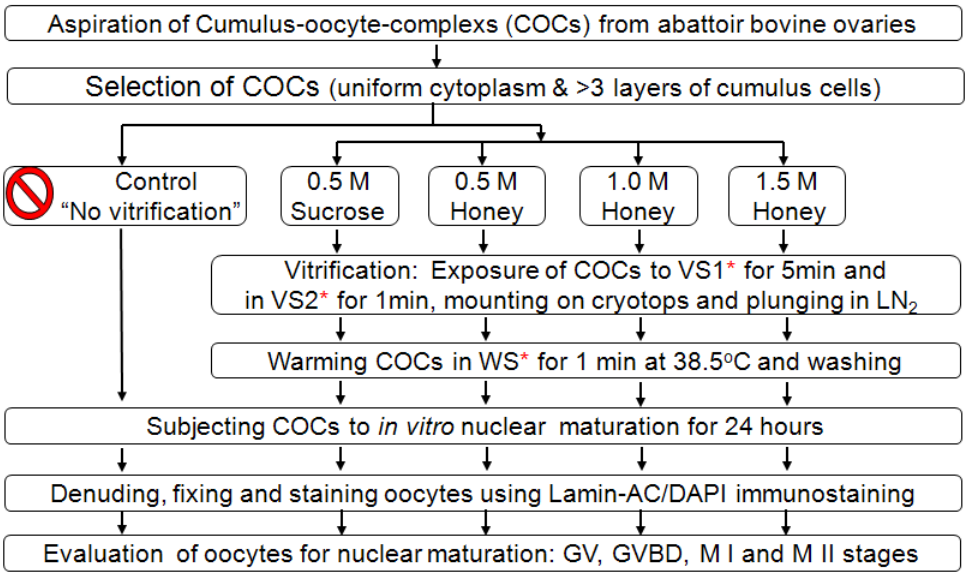

Figure 4.2 Experimental design to study the effects of vitrification of bovine oocytes using natural honey or sucrose as non-permeant cryoprotectants on their subsequent *in vitro* maturation. VS1* = equilibration solution (TCM-199 + 7.5% EG + 7.5% DMSO + 20% CS). VS2* = vitrification solution 2 (TCM-199 + 15% EG + 15% DMSO + 0.5M sucrose or 0.5M, 1M or 1.5M honey + 20%CS). WS* = warming solution (TCM-199 + 0.5M sucrose or 0.5M/ 1M/1.5M honey + 20% CS).

4.3.8.2 Experiment 2:

This experiment was designed to investigate *in vitro* fertilization, and rates of cleavage and blastocyst formation of GV-bovine oocytes vitrified in a medium containing 0.5M sucrose (17.1% w/v) vs. 1.0M (21.7% w/v) natural honey. An outline of this experiment is illustrated in

Figure 4.2. Briefly, COCs were distributed randomly into three groups including control (not vitrified) and vitrified groups using sucrose and honey. The COCs were vitrified (as described above in section 4.3.4) using 1.0M honey vs. 0.5M sucrose as non-permeating cryoprotectant in vitrification medium. After 24 h of *in vitro* culture, COCs groups underwent *in vitro* fertilization and culture. In this experiment, six replicates were performed.

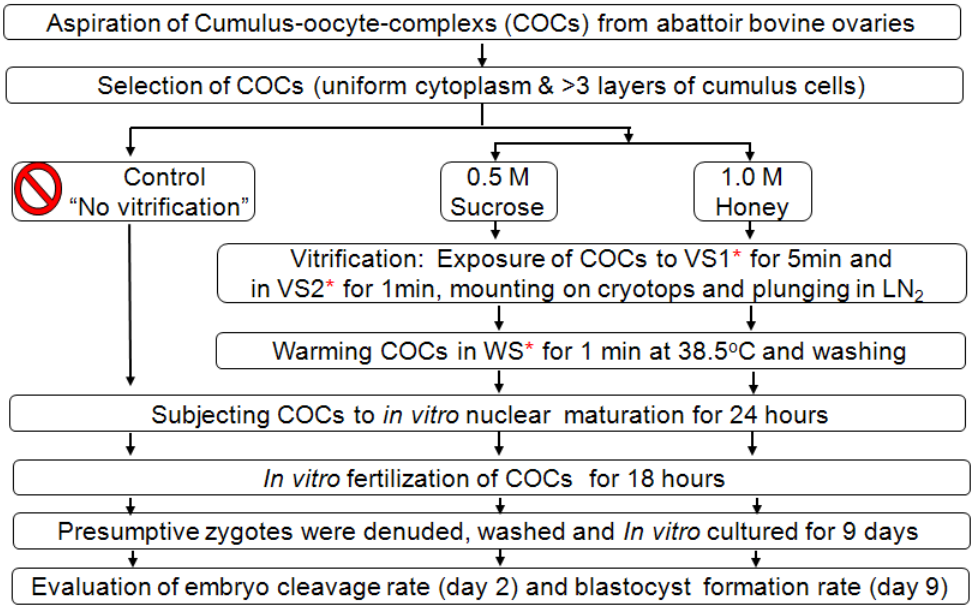

Figure 4.3 Experimental design to study the effects of vitrification of bovine oocytes using natural honey or sucrose as non-permeant cryoprotectants on their subsequent *in vitro* fertilization, and cleavage and blastocyst rates. VS1* = equilibration solution (TCM-199 + 7.5% EG + 7.5% DMSO + 20% CS). VS2* = final vitrification solution (15% EG + 15% DMSO + TCM-199 + (0.5M sucrose) or (0.5M, 1M or 1.5M honey) +20%CS). WS* = warming solution (TCM-199 + (0.5M sucrose) or (1M honey) + 20% CS).

4.3.9 Statistical analysis

Data are presented as means ±SEM. In experiment 1, differences in maturation rate among treatment groups were analyzed using one-way analysis of variance followed by post hoc analysis with the Fisher's protected least significant difference (LSD) test. In experiment 2, the

statistical analysis of embryo development data was carried out using chi-square (Fisher's exact test for the blastocyst formation rates). The differences between treatment means were considered significant at a level of $P<0.05$. Data were analyzed using SAS® Enterprise Guide 4.2 (SAS, Cary, NC, USA).

4.4 Results

In experiment 1, *in vitro* maturation ability of non-vitrified (control) and vitrified COCs in 0.5 M sucrose or different concentrations of honey was determined. The rate of oocyte maturation (MII stage) was significantly higher in control group than vitrified groups ($P=0.0003$; Figure 4.4). On the other hand, no significant differences were dedected in nuclear maturation rates among vitrified COCs groups ($P>0.05$; Figure 4.4).

In experiment 2, the ability of control and vitrified COCs to undergo cleavage and early embryonic development was investigated (Figure 4.5). Cleavage rates were significantly higher ($P<.0001$) in the control group than in vitrified groups. However, the cleavage rates among vitrified groups were not significantly different ($P=0.0723$; Figure 4.5). The blastocyst formation rates were significantly higher ($P<.0001$) in the non-vitrified (control) group than in the vitrified groups; however, blastocyst formation rates in the honey group were significantly higher ($P=0.0036$) than in the sucrose group (Figure 4.5).

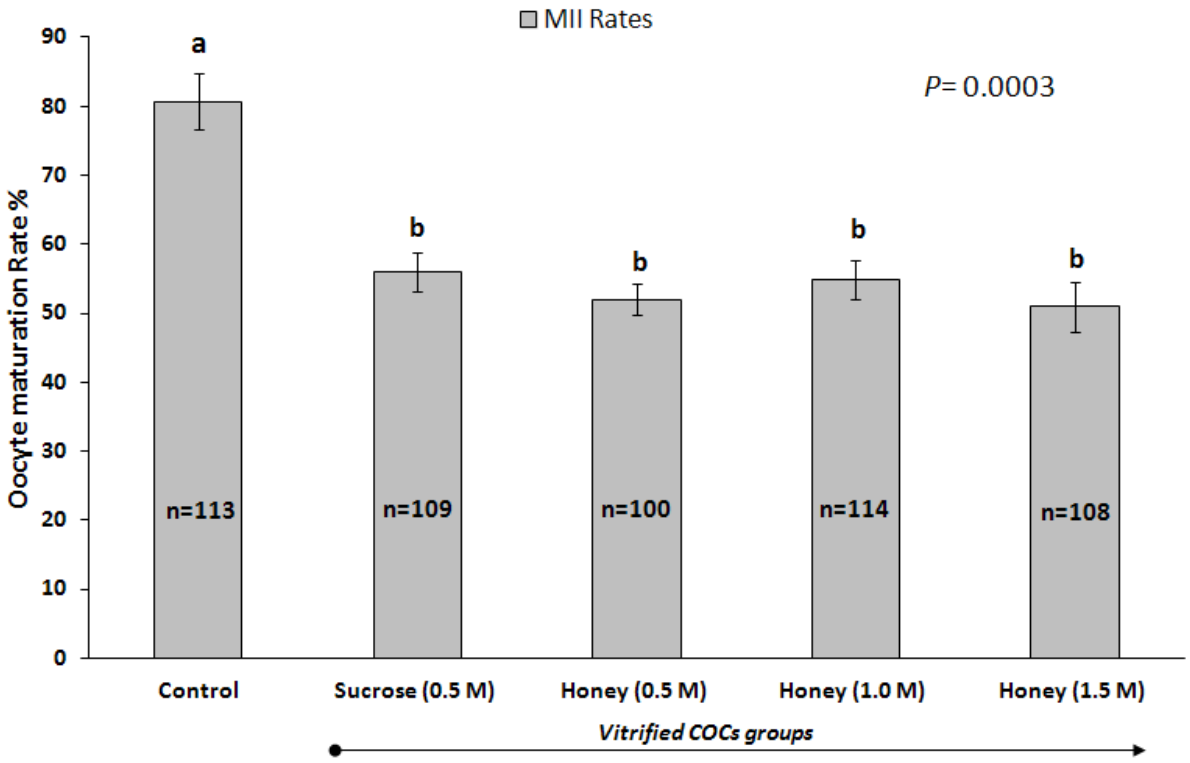

Figure 4.4 Nuclear maturation of bovine COCs (GV) following vitrification in medium containing honey or sucrose. Oocytes that have reached metaphase II (MII) stage were considered fully matured. Each bar represents mean ± SEM, and different letters indicate significant difference among groups (P<0.05).

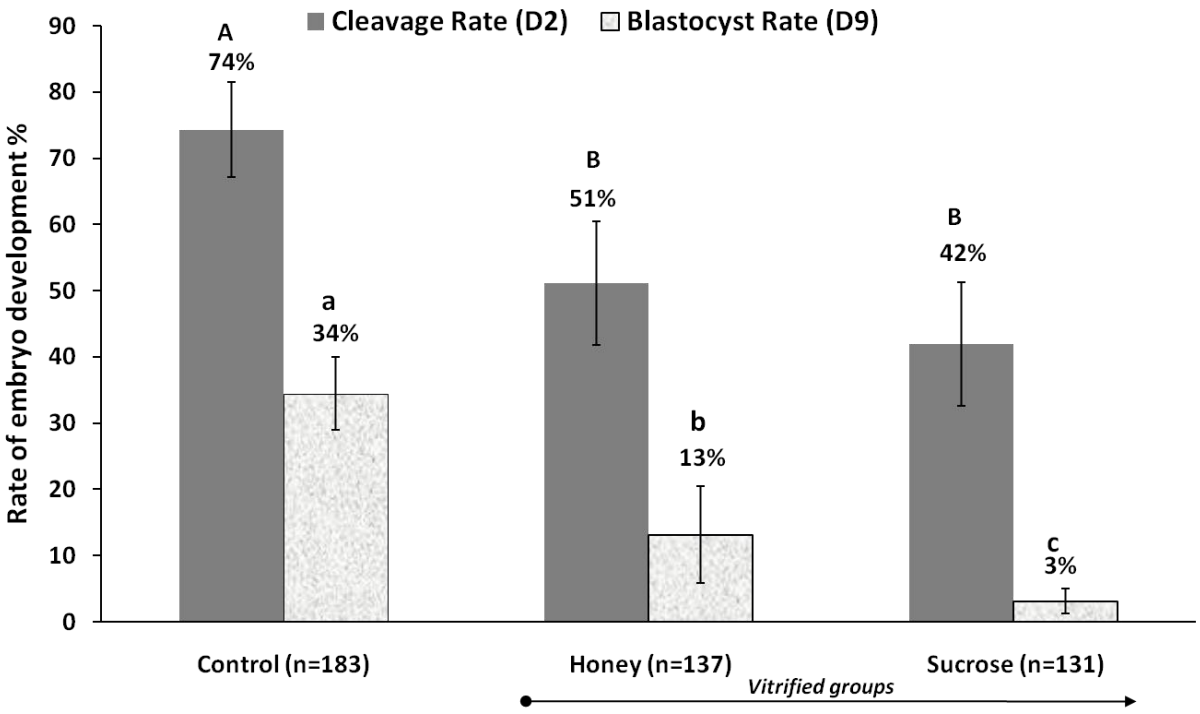

Figure 4.5 Effects of natural honey or sucrose as non-cryoprotectant in vitrification solution on post-thaw *in vitro* cleavage and blastocyst production rates of bovine oocytes. Each bar represents mean ± SEM, and within cleavage or blastocyst rate, different superscripts indicate a significant difference between groups ($P<0.05$).

4.5 Discussion

In this study, we tasted the hypothesis that natural honey can function as nonpermeating cryoprotectant in vitrification medium and can improve the post-warming viability of immature bovine oocytes (GV). Our study demonstrated that COCs vitrified whether in medium containing honey or sucrose had similar maturation and cleavage rates (Figure 4.4 &4.5). In addition, the ability of vitrified/warmed oocytes to develop and form blastocysts in the honey group was markedly higher than in the sucrose group (Figure 4.5). Finally, hatching blastocyst occurred only in honey group (data not shown).

In this study, the sucrose group was considered to be the second control since it is the most commonly used sugar in vitrification medium at a concentration of 0.5M. Our results showed that maturation, cleavage and blastocyst formation rates of 0.5M sucrose groups were comparable to other studies of vitrification of bovine GV oocytes using the cryotop® and OPS methods [17,170,204]. Our's (sucrose group) and previous results indicate a low relationship between cleavage and embryonic (blastocyst) development rates. In a recent-cytotoxicity study in oocytes, the cleavage rate was not considered a reasonable parameter for evaluating the oocytes' viability because the adverse toxic effects are reflected on later stages of embryonic development rather than early cleavage stages [205]. Based on this, blastocyst formation rate is the satisfactory parameter for evaluation of post-thaw viability of vitrified oocytes.

In this study, the honey group had a higher blastocyst rate compared to the sucrose group (13.1 vs. 3.1%; Figure 4.5). The higher rate of blastocyst formation in the honey group might be due to relatively better dehydration of the COCs prior to freezing. In our previous study (chapter 3), we found that honey (1M) based media provided better oocyte dehydration than sucrose (0.5M) since honey-based medium withdrew approximately 10% more of intracellular active water than sucrose-based medium (as we discussed in chapter 3, section 3.5, that volume of intracellular-free water averages 75% of the original volume of bovine oocyte). It was found that an inadequate cell dehydration leads to formation of large intracellular-ice crystals, which can be lethal to the cell [206]. We speculate that COCs dehydrated by 1M honey-based medium were able to undergo more dehydration than COCs dehydrated in 0.5M sucrose-based media, and therefore, sustained lower intracellular oocyte damage during cooling and warming procedures.

From another point of view, natural honey along with predominant saccharide components contains a great number of bioactive substances such as organic acids, enzymes,

antioxidants and vitamins. Such a unique composition provides numerous nutritional, biological and pharmacological effects in living cells [42-44,48-50,53]. Therefore, we can speculate that such wide variety of bioactive substances in natural honey might be responsible for the better post-warming blastocyst formation rate. As honey is composed of a wide variety of amino acids (AAs) [43,44,54], some of the amino acids including glycine and alanine have proven to help stabilizing the cell membrane from freezing by stabilizing the phospholipids of cells [207]. In addition, recent studies have stated that AAs have been successfully used as non-permeating cryoprotectant for mammalian cells including sperm and oocytes [19,55,56]. It was demonstrated that addition of glutamine (one of AAs in honey) in vitrification medium improved maturation ability of vitrified/warmed immature bovine oocytes [19].

In summary, this study demonstrated that vitrifying oocytes in medium containing 1M (21.7%; w/v) of natural honey is more successful than commonly used sugar (0.5M sucrose) in terms of long-term, post-warm oocyte viability and embryonic development. Further study is required to investigate the effects of treating oocytes with natural honey before and after vitrification procedure and to elucidate the exact mechanisms by which honey mediates these beneficial effects.

CHAPTER 5: GENERAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

As described in chapters 2 and 3 of this thesis, the limited success in post-warming viability of vitrified oocytes requires emphasis on minimizing the effects of two fundamental problems, including intracellular ice crystallization and cytotoxicity due to high concentrations of CPs. Therefore, the overall task of this study was to reduce the chance of intracellular formation of ice crystals during cooling and warming procedures while minimizing the cytotoxic effects of CPs in an attempt to improve post-warming viability and subsequent embryonic development of vitrified bovine oocytes.

Our study has focused on decreasing the effects of intracellular ice crystalization by dehydrating oocytes sufficiently prior to vitrification using natural honey as a non-permeant CP in order to improve viability of vitrified bovine oocytes (COCs). In this study we investigated that natural honey as non-permeant can provide cryoprotective functions of sugars that have been used in the vitrification medium. Our study demonstrated that honey and sucrose-based media have a similar effect on the volumetric changes in matured bovine oocytes (chapter 3, Figure 3.3 & 3.4), and their volumetric changes are dependent on the concentrations of the honey and sucrose and time (Figure 3.5). As we expected, the ability of honey-based medium in oocyte dehydration was based on the fact that natural honey in its dry matter comprises 95 to 97% sugars [42,43,208] which are mainly fructose and glucose, i.e. 75-85% of the total sugars in honey [208].

Our criteria for selecting appropriate concentrations of sucrose and honey was based on the shrinking volume at which oocytes can obtain maximum dehydration without any physical damage. The intracellular water consists of free water and bound water. The bound water

molecules of the oocytes are critically important to protect the structure of the intracellular molecules and organelles to maintain their functions [41]. Therefore, a delicate balance of water contents must be maintained during dehydration of the oocytes. In previous studies, the osmotically inactive volume of MII bovine oocytes in the presence of NaCl [115] and sucrose [199] was determined to be 25% and 26% respectively, of the isotonic cell volume. This means that volume of intracellular-free water averages 75% of the original volume of bovine oocyte. However, it is speculated that dehydration of oocytes at this level is harmful since it requires too long an exposure time (>3 min) to the toxic effects of CPs in VS2. A recent study has determined that the osmotically inactive volume of MII human oocytes was found ~19% [200] , but the osmotic tolerance limit in human oocytes was suggested to not exceed 57% of the isotonic oocyte volume [40]. Our study shows that bovine oocytes in the concentration of 0.5M sucrose and honey-based media require 60 sec to reach 54% and 55% of the isotonic cell volume, respectively (chapter 3; Table 3.2).

An ideal vitrification solution should have a maximum sugar concentration in order to enhance cell dehydration, thus minimizing the quantity of intracellular permeant CPs while not exceeding the osmotic-tolerance limit of oocytes [28]. In this way, sugar alleviates high concentrations of penetrating cryoprotectants, and thus decreases their toxicity [40,41]. Our study found that if sucrose-based medium increased from 0.5M (commonly used concentration) to a higher concentration (1M or higher) in order to enhance oocyte dehydration, a faster oocyte dehydration was observed in ≤30 sec, compared to 60 sec by using 0.5M sucrose (chapter 3; Table 3.2). This fast shrinkage of oocyte volume might lead to mechanical damage to oocytes. When a higher sugar concentration in vitrification medium is desired in order to safely enhance oocyte dehydration prior to cooling, 1M honey (21.7%; w/v) is suggested here. Because honey

consists mainly of fructose and glucose (as mentioned in chapter 1), and these monosaccharides have a lower viscosity than disaccharides [36]. This concentration of honey can enhance oocyte dehydration to 46% of its original volume in 60 sec, and these dehydrated oocytes were observed to regain full rehydration in 60 sec (Table 3.2). We determined that dehydrating denuded-MII oocytes in 1M honey-based-vitrification medium reaching a level of 46% of its original volume in 60 sec is adequate and safe. A recent study has investigated the osmotic tolerance limits of GV bovine oocytes, suggesting that the safe reduction in volume is 46% of their original volume [199]. This volume reduction is compatible with the one (48%) that can be achieved with 1M honey. We speculate this level of oocyte dehydration (48%) and reasonable exposure time (60-90 sec) can minimize the chance of IIF and limit the exposure to toxic effects of CPs. However, in case of dehydrating GV COCs, we suggest prolonging the dehydration time up to 90 sec because oocytes at GV stage have less membrane permeability compared to MII stage. Also, cumulus cells retard the movement of water and CPs between the extra and extracellular space (as explained chapter 2, section 2.6).

In the second study (chapter 4), it was hypothesized that natural honey (unheated) can function as a nonpermeant CP in vitrification medium and can improve the post-warming viability of immature bovine oocytes. The optimal concentration of honey (1M or 21.7%) (suggested from dehydration study, chapter 3) was used in vitrification medium in order to investigate the post-warming viability of vitrified COCs. We expected improvement in viability of vitrified COCs due to safe and sufficient oocyte dehydration occuring prior to vitrification using (1M or 21.7%) honey.

Our results of this study have demonstrated that COCs vitrified in a medium containing honey or sucrose resulted in similar maturation and cleavage rates (Chapter 4; Figure 4.4); however, the blastocyst formation rate of oocytes vitrified in a medium containing natural honey was significantly higher compared to sucrose (Chapter 4; Figure.4.5), and the hatching of blastocysts occurred only in the honey group (data not shown). Sucrose (0.5M) was included as a second control for the vitification of oocytes. Our results showed that the rates of maturation, cleavage and blastocyst formation from bovine COCs in the sucrose group were in agreement with other studies [17,170,204] using open cryodevice methods (cryotop® and OPS). These studies reported low blastocysts formation rates similar to our study in the sucrose group. This low blastocyct formation in sucrose group might be due to insufficient oocyte dehydration by the sucrose medium, whereas the substitution of sucrose with honey markedly improved blastocyst formation. In our dehydration study (chapter 3) we found that 1M honey based medium provided better oocyte dehydration than sucrose (0.5M) since honey withdrew ~10% more intracellular active water compared to sucrose. It was found that inadequate cell dehydration leads to the formation of large intracellular-ice crystals, which can be lethal to the cell [206]. This minimized the chance of oocyte damage by intracellular ice crystallization during cooling and warming procedures. In addition, this superior reduction in the oocyte volume by the honey-based medium prior to vitrification means a superior reduction in the quantity of the CPs within the oocytes, so that the toxic effect is decreased [27,28]. Therefore, the chance of forming intracellular ice crystals and toxic effects were probably less in oocytes that were vitrified in a medium containing 1M of honey compared to 0.5M sucrose.

Taking into account the finding in our dehydration study (chapter 3), the minimum time required for bovine oocyte to attain dehydration in 0.5 M sucrose-based medium was 60 sec and

for regaining almost full rehydration was 90 sec (chapter 3; Figure 3.4). But this time was based on using denuded oocytes not COCs. However, many vitrification protocols applied to bovine oocytes, including our protocol, expose COCs to final vitrification solution for only 30 sec up to ≤ 1 min [17,170,204]. Our findings show that exposure of less than 60 sec is not adequate for oocytes to attain sufficient dehydration. Therefore, a little extra time might be required for sufficient dehydration.

The dehydration of cells to avoid the risk of cytotoxicity from prolonged exposure time to the final CPs before plunging into LN_2 is a critical for post-warming cell viability and their subsequent embryonic development [209]**.** There is no ideal cryoprotectant used in cell cryopreservation that is free of toxicity [132]. It was demonstrated that the post-warming viability and embryo development rate after exposing cells to CPs (mixture of 25% DMSO and 25% EG) followed by vitrification was high only up to 1.5 min of exposure, and decreased after 2 min [210]. Therefore, we suggest extending the exposure time of bovine oocytes in the vitrification solution from 60 sec up to ≤90 sec at RT. Such exposure time is adequate for oocytes (immature COCs) to attain sufficient dehydration prior to vitrification. The suggested time of exposure (60-90 sec) would be enough for oocytes to avoid the formation of large intracellular ice crystals [28] and the cytotoxicity of CPs [210]; otherwise, they lead to detrimental effects on subsequent post-warming oocyte viability.

The warming time of oocytes is equally important as the cooling procedure in order to avoid the detrimental effect of intracellular ice recrystallization and to minimize the cytotoxic effects of permeating CPs which limit post-warming oocyte viability and subsequent embryonic development [132]. In our study we used 60 sec as a given time for the warming of vitrified

COCs. However; we found that dehydrated oocytes in a 0.5M sucrose-based medium require at least 90 sec as a given time in order to regain their full rehydration (chapter 3; Figure 3.4). In other words, the warming procedure for vitrified oocytes in a medium containing 0.5M sucrose might not be adequate to dilute and withdraw the permeating CPs; thus there is risk of the cytotoxic effects on post-warming oocyte viability and subsequent embryonic development.

In addition to the greater dehydration of COCs in honey-based medium, we speculate that vitrifying and warming COCs in media containing natural honey provides a wide variety of bioactive beneficial substances to oocytes. Some of these honey components (mentioned in chapter 2&3) function as antioxidants, including various phenolics, peptides, organic acids, vitamins and enzymes (such as glucose oxidase, catalase and peroxidase), and they possess synergistic interactions resulting in a stronger antioxidant capacity [47,57]. These antioxidatnt components present in honey might be responsible for the improved post-warming blastocyst formation rate of COCs that vitrified and warmed in media containing natural honey.

In this study, the increased rate of blastocyst formation of vitrified/warmed oocytes using honey (13%) as compared to sucrose (3%) indicates that natural honey can be substituted for sucrose as a non-permeating CP in the vitrification medium. Therefore, the use of natural honey (1M or 21.7% w/v) will result in improvement of *in vitro* production of embryos to enhance female gamete banking. Oocyte banking is achievable when vitrified/warmed oocytes can be fertilized and successfully developed into high quality embryos capable for embryo transfer (ET), a product which might be utilized in future to re-establish any loss of animal genetic diversity. In regards to its application in industry, it is speculated that vitrification of oocytes in the honey-based medium might be more successful for in vitro production of embryos, leading to

a higher pregnancy rate. However, the prospective use of natural honey as a non-permeating CP in the cryopreservation medium might encounter challenges to replicate these results due to variability in sources of honey production around the world (i.e. variation in honey composition depends on the botanical origin). In fact, standardization of natural honey needs further investigation in order to confidently use this product for cell/tissue vitrification.

Future directions

The first line of study proposed here is the modification in the existing vitrificatuion protocol. The results of this thesis give rise to many interesting thoughts for future studies. The first future study should focus on optimizing time intervals in the vitrification protocol by examining long term viability of oocytes that vitrified-warmed in VS2 containing 1M honey:

First, COCs exposure time in equilibration solution (VS1) should be reduced to the minimum to avoid potential cytotoxicity to the oocytes. Our existing vitrification protocol includes COCs' equilibration in VS1 for 5 minutes. This time can be decreased to **1 min** only as permeating CPs can penetrate the cell in less than 1min.

Second, adjusting the exposure time of COCs in vitrification 2 (VS2). The widely used vitrification protocol includes adding COCs to VS2 for $\leq$ 1 minute. Based on our results, the exposure time should be prolonged to 60-90 sec. This is because sufficient dehydration requires at least 60 sec when denuded oocytes are used, but if COCs is the choice to undergo vitrification, the exposure time should be prolonged. Therefore, it is speculated that 90 sec exposure in VS2 is sufficient for dehydration. The overall exposure time for introducing COCs in VS1 and VS2 should be limited to approximately 2.5 to 3 min.

Third, the warming time for vitrified COCs in our existing vitrification protocol is 1 min, and it is hypothesized that this is not sufficient for removal of CPs. The time for COCs rehydration and removal of the CPs should be extended between 90-120 sec, based on the dehydration times found in this study, and it should be done in a stepwise manner (1M, 0.5M and finally in 0.25 M). The extended warming time (90-120 sec) would be sufficient for rehydrating vitrified COCs as well as to remove intracellular CPs leading to minimizing their cytotoxic effects on viability.

Optimizing our vitrification protocol including adjusting the exposure time of COCs in VS1 (60 sec), VS2 (90 sec) and warming solution (90-120 sec) would be enough for vitrified COCs to avoid the formation of intracellular ice crystals [28] with minimum of cytotoxic effects of the CPs [210], and the post-warming oocyte viability and subsequent embryonic development should improve. After optimization of the vitrification protocol of natural honey-based media, the investigations on vitrification of oocytes/embryos of other farm-animal species should be carried out.

The second line of future study should investigate the incorporation of natural honey in *in vitro* culture system including medium for IVM, IVF and IVC since honey has the nutritional, biological and pharmacological effects on cell viability. One suggestion, for instance, is to investigate the effects incubation of oocytes (before and after vitrification) in a medium containing low levels of honey (i.e 1, 2 or 3% of honey added in TCM) for 1 h (particularly if using immature COCs) as they will accumulate energy to tolerate cooling stress and injury during vitrification procedure, and to utilize it during IVM and IVF. These bioactivities of the natural honey in living cells might counter the lethal cryo-injiry and cytotoxic effects of CPs on

the stressed vitrified oocytes/embryos. Furthermore, honey contains a wide variety of components that have the capability to serve as antioxidants, including phenolics, peptides, organic acids; enzymes, vitamins (vitamin C being the most dominant), and these components as a group provide a combined antioxidant activity in synergistic interactions [57]. For instance, a remarkable improvement in bovine blastocyst survival was observed when anti-oxidants were added to the culture media following vitrification [1,211].

The third line of future study is to investigate utilizing the anti-microbial activity of natural honey to counter the possibility of the microbial cross-contamination that resulted from the direct contact of the LN_2. This microbial cross-contamination is still an open problem in the in vitro production of embryos using oocyte/embryo vitrification. Although several methods of closed-system vitrification have been developed such as cryotip, cryohook etc. to solve this problem [197], unfortunately, vitrification by using a closed system has failed to demonstrate the same efficiency of high cooling/warming rates as obtained from an open system (direct contact to LN_2). To date, the open system, despite the biosecurity risks, is still the indispensable option to achieve the required cooling and warming rates for very sensitive samples like oocytes [94]. One of the factors responsible for the anti-microbial property of honey is due to the the presence of glucose oxidase enzyme. This enzyme is inactive status when the honey is in full density and becomes active when honey is diluted, producing hydrogen peroxide and gluconic acid from glucose [212]. Therefore, it would be beneficial to investigate the anti-microbial property of 1M (21.7%) honey-based medium in preventing the cross-contamination that resulted from the direct contact between LN_2 and vitrified oocytes (or embryos).

Conclusions

1. Natural honey, as a rich mixture of sugars, has similar dehydration effects on bovine oocytes as sucrose (commonly used) in vitrification media, and the volumetric response of oocytes during dehydration and rehydration in honey- and sucrose-based media is concentration dependent.
2. Natural honey 1M (21.7% w/v) was found to be a suitable concentration of honey that can be used in the vitrification medium. A better blastocyst formation rate was observed when bovine oocytes (COCs) were vitrified and warmed in a medium containing natural honey (13%), as compared with sucrose (3%).
3. The superiority of honey over sucrose might be due to: (a) sufficient oocyte dehydration in honey vitrification-based medium (1M) that withdrew 10% more intracellular active water compared to sucrose (0.5M); and (b) the nutritional substances present in the honey-based medium that benefit the COCs.
4. For proper dehydration of bovine COCs prior to vitrification, we recommend using 1M (21.7% w/v) natural honey with an exposure time of 60-90 sec. However, optimizing the current vitrification protocol using sucrose for better dehydration of bovine COCs before vitrification, we recommend extending the exposure time of COCs in VS2 to 90 sec when using 0.5M sucrose ; otherwise, increasing sucrose concentration up to 0.75M with an exposure time of 60 sec.

In conclusion, our study has focused on decreasing the effects of intracellular ice crystalization as well as the cytotoxic effects of CPs. This could be achieved by sufficiently dehydrating oocytes prior to vitrification, using natural honey as a non-permeant CP, in order to

improve the post-warming viability of vitrified oocytes (COCs). The honey-based media has proven its ability to cause dehydration and rehydration in bovine oocytes sufficiently and safely during vitrification. As a result, cryo-damage and the quantity of CPs in intercellular space can be significantly minimized, thus reducing intracellular ice formation and lessening the cytotoxic effect of CPs, leading to an improved post-warming viability and subsequent embryonic development of vitrified bovine oocytes. Therefore, the hypothesis in this thesis was confirmed that “natural honey” as a bioactive mixture of mainly sugars can be used as a non-permeant CP in the vitrification medium of bovine oocytes (COCs).

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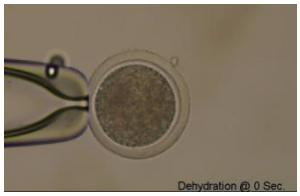
APPENDIX

1. Dehydration of ooplasm bovine oocytes in 0.5M honey-based medium:



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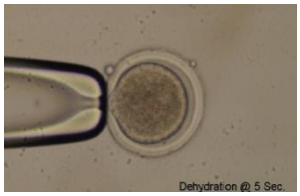

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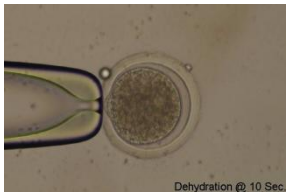

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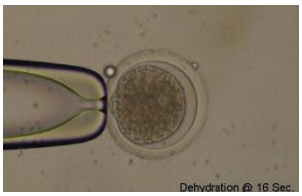

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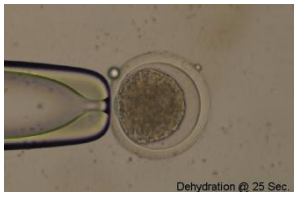

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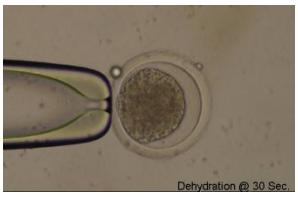

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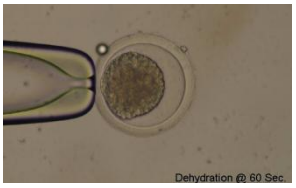

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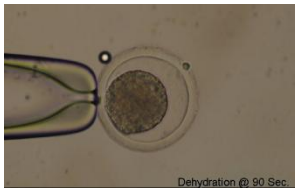

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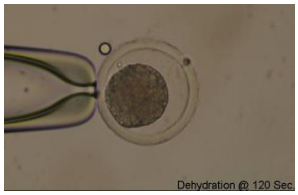

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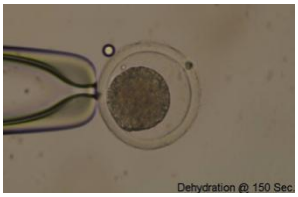

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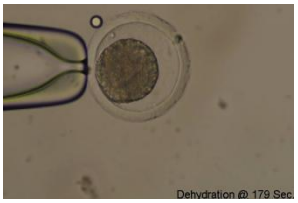

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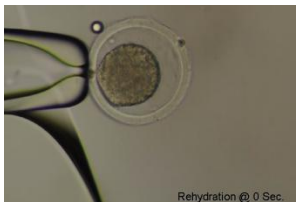
2. Rehydration of ooplasm bovine oocytes in the control medium (TCM + 5% CS):



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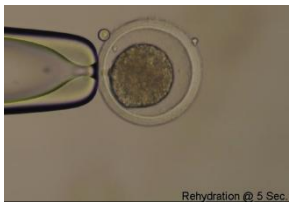

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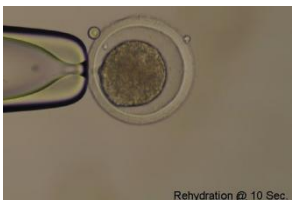

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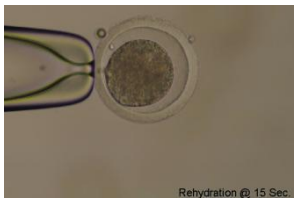

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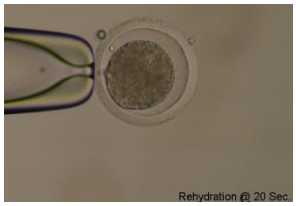

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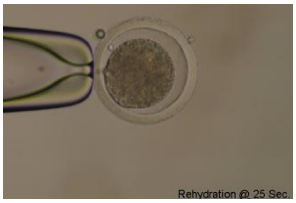

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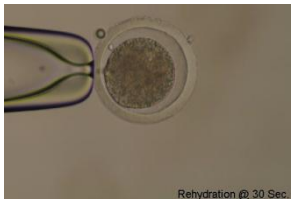

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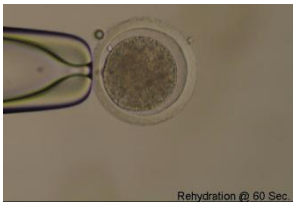

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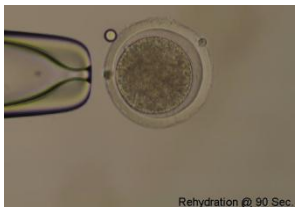

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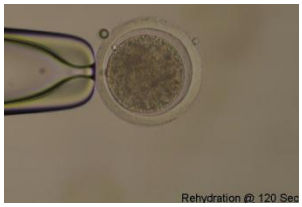

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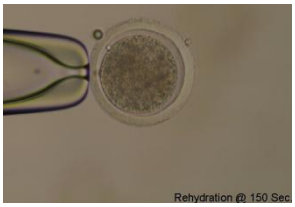

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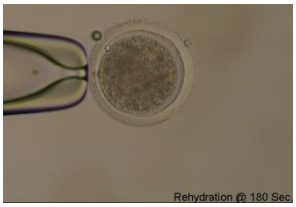

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